THE AQUACULTURE AND BIOLOGY OF ‘OPIHI ‘ALINALINA (*CELLANA SANDWICENSIS*)

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Dedication

This dissertation is dedicated to my family, friends, and colleagues whom have inspired me to be a change-maker in our community.

I specifically want to dedicate the completion of graduate school to my grandmother Laura, my mom Patricia, and my father Barry – whose unconditional love and financial support made it possible to attend Institutions like Punahou School, University of San Diego, and University of Hawai'i.

"To whom much has been given, much will be required." (Luke 12:48)

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Abstract

The present paradigm in global seafood production suggests that the market demands farm-raised seafood over wild-caught seafood. Whether this is due to a shift in market preference or simply an overall increase in farm production, there is an overwhelming amount of empirical evidence for diminishing return of wild stocks. For this reason, development of high-value mollusc species remains a top priority for the aquaculture industry – the ultimate goal being to improve food-security and reduce harvesting pressure on wild fisheries.

For selection of new aquaculture species, it's critical to understand the life-history to effectively engineer infrastructure, establish technical protocols, and optimize nutrition for production. In Hawai'i, 'opihi 'alinalina or yellowfoot limpet (*Cellana sandwicensis*), a prized mollusc shellfish and significant biocultural resource, has been a prospect aquaculture species for many years; and recently, there has been progress made towards closing the life cycle of *C. sandwicensis* in captivity.

In this dissertation, the primary research objectives were to i) develop a suitable formulated feed by examining the effect of protein to energy ratio on growth performance of adult yellowfoot limpets in aquaculture; ii) establish suitable methodologies for controlled spawning and larval rearing to settlement; iii) examine the effects of a novel synthetic GnRH-like peptide hormone on limpet reproduction utilizing proteomics and solid-phase-peptide-synthesis; and iv) define the growth of yellowfoot limpets utilizing sclerochonology and stable isotope analysis by secondary ion mass spectrometry.

The current advances of aquaculture technology and biology of *C. sandwicensis* includes: the fabrication and implementation of state-of-the-art recirculating aquaculture systems for broodstock, settlement, and grow-out; testing of a suitable formulated feed that meets nutritional

requirements of the species; development of natural and hormone-controlled spawning techniques using a novel limpet GnRH-like peptide; establishment of methodology for larval rearing to settlement; and the first discoveries of key life-history traits.

This research collective closes the present gap-in-knowledge pertaining to *C. sandwicensis* in aquaculture, and its biology – development, growth, and reproduction. Future research and development will focus on the settlement of limpets and the underlying mechanisms influencing survival in aquaculture, as well as to support ‘opihi fisheries management through understanding the life-history of *C. sandwicensis*.

List of Publications

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Chapter 1

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Chapter 2:

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Chapter 3:

Mau A, Fox K, Bingham JP (2017) The Reported Occurrence of Hermaphroditism in the Yellowfoot Limpet (*Cellana sandwicensis* Pease, 1981). *Annals of Aquaculture Research*, 4, 1045.

Chapter 4:

Mau A, Bingham JP, Soller F, Jha R (2018) Maturation, spawning, and larval development in captive yellowfoot limpets (*Cellana sandwicensis* Pease, 1861). *Invertebrate Reproduction and Development*, 62, 239-247.

Submitted Manuscripts:

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Chapter 6:

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Conference Proceedings:

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Table of Contents

List of Tables

Page

Chapter 2

Chapter 4

Chapter 5

List of Figures

Introduction

The U.S. aquaculture industry is small in comparison to the current global market. The U.S. has strict environmental and business policies that make aquaculture production a serious challenge, especially in working coastal fronts, which ultimately contributes to a seafood trade deficit that equals to billions of dollars.

An area where there has been success in the U.S. Aquaculture Industry is sustainable, high-value shellfish that provide ecosystem services while off-setting wild capture fisheries (i.e. oysters, clams, mussels). The U.S. has also succeeded at restoration aquaculture of endangered species of abalone along the California coast. While the U.S. Aquaculture Industry is both ecologically and economically driven, research must also address the biocultural significance of certain species that coastal communities and subsistence fishers are highly dependent on.

In Hawai'i, the endemic group of marine limpets referred to as 'opihi (*Cellana spp.*) is considered the most valuable shellfish in the wave-exposed rocky intertidal. At the turn of the 19th century, threatening population declines around the State brought concern to this commercial- and subsistence-based intertidal fishery. Despite not accounting for the large subsistence 'opihi fishing effort, available commercial 'opihi catch landings reported by the Hawai'i Department of Land and Natural Resources suggest that wild-stocks are declining year-to-year, with State-wide annual harvests of ~9,000 kg (shell on). As far as market availability, this shellfish can be purchased year-round at select fish markets, selling at US$10/kg (shell on), as well as online via black-markets. With increased supply from aquaculture production, we reduce some of these problems associated with a declining fishery.

In the early 1980's, in response to this dire issue, researchers embarked on their attempt to culture 'opihi. As part of Hawai'i Department of Agriculture's Aquaculture Development Program (ADP), they released a report titled *'Opihi. Their Biology and Culture*. Included in this report were both field and laboratory studies on the reproductive biology of *C. exarata* and *C. sandwicensis*, which, to this day, maintains implications for both fisheries management policies and establishment of a new aquaculture sector.

Although this report contributes valuable insight into possibilities of culturing 'opihi, researchers should question if this work is entirely possible given the lack of information regarding aquaculture systems, nutrition and feeding, and practical propagation methodology. And generally speaking, there are few studies pertaining to the aquaculture of limpets, a prospective area of research that is of interest around the world.

The focus of this dissertation is the aquaculture and biology of the preferred Hawaiian limpet species, 'opihi 'ainalina (*Cellana sandwicensis*). The overarching goal of this dissertation is to develop pilot-scale aquaculture technology for 'opihi 'alinalina. The overall research hypothesis is that *C. sandwicensis* can be cultivated in a laboratory environment, and is considered a suitable candidate aquaculture species due to their fecund nature, high-market value, and cultural significance.

In Chapter 1, this dissertation opens by disseminating background information pertaining to the aquaculture of abalone and limpets, two commercially important mollusc shellfish groups. In this comprehensive review of the current literature, we aim to reveal knowledge-gaps and open discourse for areas of research in limpets. To the best of our knowledge, there are no commercial limpet farms, and therefore, we justify the exploration into the present culture methods/systems and animal nutrition established for abalone as a parallel model to limpets.

Next, the dissertation includes a series of studies that examine the feasibility of cultivating limpets in captivity under controlled conditions, as well as provides important findings that will fill the gap-in-knowledge pertaining to species biology (i.e. growth, development, and reproduction) deemed important for aquaculture.

In Chapter 2, we aim to formulate a suitable commercial feed based on preliminary studies of abalone and limpet. In this nutrition study, the objectives are to develop, fabricate and implement a novel grow-out system, and to evaluate the effect of varying protein to energy (P:E) ratio diets on growth performance of limpets. This study not only includes animal husbandry of limpets, but also overcomes issues associated with producing and feeding natural diets (i.e. biofilm and crustose coralline algae).

In Chapter 3, we report the first occurrence of a hermaphrodite yellowfoot limpet. This chapter tackles a phenomenon observed for wild yellowfoot limpets, where sex ratios change across time and space, and considers the species' reproductive strategy. This sets up strategies for the next chapter, which depends upon wild-collected limpets for controlled reproduction. Although it is impossible to sex this species without sacrificing animals, we will assume sex-ratios to be near 1:1 M:F ratio during spawning season for the purpose of aquaculture.

In Chapter 4, we aim to develop hatchery technology for seed production of limpet. The objectives of this chapter are to develop baseline methods for maturing limpets in captivity, spawning, and larval rearing, as well as to describe early development through metamorphosis. Although we applied hormone control to successfully spawn limpets, the effects of the hormone are unclear and warrant further testing in the next chapter. This would give us control over seed production in the hatchery.

In Chapter 5, we aim to examine the effect synthetic GnRH analogies on reproduction in *C. exarata* and *C. sandwicensis* for evaluating current controlled propagation methods. The objectives were to identify, synthesize, and test a novel GnRH variant based on phylogenetic analysis, and to test administration methods – hormone injection and hormone exposure – in a side-by-side bioassay to determine if these GnRH isoforms have multiple modalities. This is the first study looking into the endocrine system of a limpet model, and gives us important functional considerations for development of novel spawning technology.

In Chapter 6, er aim to reconstruct the life-history of *C. sandwicensis* from shells of modern and historical (archived) specimens to understand how growth changes through time. The objectives were to develop the first near-daily spatial scale tropical intertidal climate proxy using secondary ion mass spectrometry (SIMS) analysis of oxygen isotopes, and to determine seasonal growth and longevity of the species. This chapter provides answers to important biological questions (i.e. *how long do limpets live? how fast to limpets grow?)* using precise analytical methodology, and establishes a growth-curve that can be applied in both aquaculture and fisheries.

Lastly, in Chapter 7, we discuss the major findings of my dissertation, and draw major conclusions on the aquaculture and biology of *C. sandwicensis*. Included in this section are implications of the presented research as it pertains to the current body of knowledge, and the future direction of ‘opihi aquaculture research and development in Hawai’i.

Chapter 1

Aquaculture of two commercially important mollusks (Abalone and Limpet): existing knowledge and future prospects

Abstract

Molluscan aquaculture produces the most food fish by volume in the global mariculture market. The historical, cultural, and ecological value of mollusks makes them a highly sought-after delicacy in many regions of the world. Specifically, the aquaculture of abalone and limpets are two key industries with striking similarities and importance within the context of food production and food security. In this review, the current knowledge of these two commercially important seafood species is explored for a comprehensive understanding of its aquaculture status. The various culture methods and systems, animal nutrition, and necessary improvements are discussed for abalone and limpets, respectively. For abalone, the knowledge base is extensive and some reviews have been written to compile essential information regarding different aspects of this healthy industry. For limpets, there is a small collection of aquaculture literature, and this is, to the authors' knowledge the first comprehensive review of the research retaining to this field. Our goal is to reveal, within the area of aquaculture research, the current status of both abalone and limpet industries while exposing their respective knowledge gaps for appropriate development of new technologies. With a firm understanding of these mollusks, the production of abalone and limpet will be sustainable for the long term.

1. Introduction

The aquaculture of mollusks is commercially important and is increasing on a global scale. The world's total production of mollusks is second to finfish with 15,170,738 metric tons (MT), annually (FAO 2014). In terms of mariculture (disregarding freshwater production), mollusk production exceeds finfish production value by over nine-hundred-fold. Despite the total mollusk production accounting for only 9.7 % of total food fish production by volume, the economic value of mollusks represented 22.4% or equivalent to US$30.9 billion, worldwide. Some countries rely heavily on their mollusk production in their aquaculture sector. For instance, Japan and Korea maintain over 50% of their total food fish production from mollusks. As wild harvested seafood becomes limited in abundance, the necessity for molluscan aquaculture is apparent.

Out of the 100,000 or so species of mollusks, only 102 are registered as commercially important species. The highly produced species are abalone, clams, mussels, oysters, sea urchin and scallops (Guo 2009). But more potential mollusk species are being researched in recent years. Limpets, a group of marine gastropods, have been harvested for human consumption for generations in coastal regions of California and Hawai'i (Erlandson *et al.* 2011; Tom 2011). The realization that some economically and culturally important food fishes will no longer be available to future generations makes aquaculture of limpets an important addition to the growing list of established mollusks.

The fate of mollusk species' success on a commercial production level is largely determined by the success of seed production (FAO 2014). To culture a new species, researchers must show the feasibility of sustaining captive bred animals at all life stages, which include the most critical and often unknown larval stage. To overcome this, adopting well-established technologies of model

mollusk species can aid in the development of protocols for rearing new species in farm environments. For this review, abalone and limpets will be considered as complimentary, commercially important mollusks because of their many similarities in biology and environmental requirements.

Currently, reviews on abalone aquaculture cover a range of topics including culture system (Aviles & Shepherd 1996), nutrition (Fleming *et al.* 1996), larval settlement (Roberts 2001), and location-based operations (Guzman del Proo 1992). However, there are no limpet aquaculture reviews to date. Henceforth, our goal is to develop the first comprehensive review on aquaculture technology of abalone and limpets for application to prospective mollusks.

1.1. Abalone

Abalone are mollusks that inhabit the tidal to sub-tidal coastlines of tropic to temperate regions. They can be found attached to hard substrates where they feed on diatoms, microalgae, and macroalgae. The genus Haliotis is comprised of approximately 90 species, 15 of which are deemed commercially important (Sales & Jansens 2004). Moreover, both wild hybrids and artificially produced hybrids have been reported to exceed 50 different crosses for genus Haliotis in the Northeastern Pacific (Geiger & Poppe 2000). The history of the seafood market for abalone reflects the decrease in productivity of wild stocks from overharvesting and poor management worldwide in conjunction with continually increasing demands. According to Cook (2016), wild landings have decreased from nearly 20,000 MT in the 1970s to less than 6,500 MT in 2015, while production of farmed abalone increased to 129,287 MT in 2015. This spike in production is due to both the illegal catches of wild abalone and the increase in aquaculture

production. The aquaculture of abalone increased from nearly 3000 MT in 2000 to around 40,000 MT in 2008 (FAO 2010). This points out the transition to and reliance on the farming of abalone to meet seafood consumption demands for this mollusk.

The largest producers of wild abalone are Australia, Japan, New Zealand, South Africa, Mexico and the United States while the largest producers of farmed abalone are China, Taiwan, South Africa, Australia, and Chile. Currently, the 300 or so abalone farms in China increased annual production from 42,373 MT to 115,397 MT from 2010 to 2015, which was attributed to a shift in using land-based farming methods to more efficient offshore sea-cage farming methods (Cook 2016). The market size of abalone are typically 100 g live whole weight, which equates to a 3-5 year grow out period (Aguilar *et al.* 2007). For the top exporting countries, the majority of their product is frozen whole and sold primarily to the Japanese market. China, however, usually prefers their abalone in the canned or dried form. The value of abalone varies widely, currently ranging from \$15-30 kg^{-1} in most of the global markets. Price discrepancies are often dependent on quality (i.e. freshness, firmness, color, and taste), size, and species available (Gordon & Cook 2010).

1.1.1. Culture systems

Abalone have been cultured using various system approaches. The two commonly used systems are land-based flow through or recirculating tanks and offshore barrels and cages. In California, both systems were implemented to increase aquaculture production during the fisheries decline in the 1970s, which was dependent on the coastal environmental parameters and access to feed resources (McBride 1998). Red abalone (*Haliotis rufescens*) and green abalone (*Haliotis discus*

hannai) were introduced to Chile as part of a plan to diversify the country's aquaculture production in 1977 and 1982, respectively. Currently, 25 farms are mainly culturing red abalone in land-based tanks, with some red abalone being cultured in sea-cage systems (restricted to the southern region) (Aquilar *et al.* 2007). Some farms choose land-based tank systems because they utilize less area while sustaining high densities of animals because farmers can manipulate the aquatic environment favorably. The ability to manipulate or control the grow-out parameters also helps farmers to overcome disease outbreak and summer mortalities. Other farms, such as those in China or Korea, may choose offshore sea-cage systems due to the reduced operating costs associated with land-based systems (Cook 2016). However, all grow-out systems depend on land-based hatcheries for their seeds or juvenile animals, which range in ages from 3-10 months post hatch. Each farm usually maintains its own hatchery and grow-out system together, but some companies operate grow-out independent from hatcheries for biosecurity measures and to reduce operational costs. Like other mollusks spawned in culture, larval abalone are reared in recirculating tanks, dump-tanks, or circular ponds using the Japanese plastic plate system for larval settlement. These plates are coated with biofilms regularly consisting of benthic diatoms and microalgae that provide reasonable nutrients for competent larvae. These post larvae live in their settlement tanks until transferred to grow-out facilities or systems.

1.1.2. Nutrition and Formulated Feeds

Research to improve the production of cultured abalone contributes positively in resolving the issue of declining wild stocks and heightening the demographics of abalone consumption. The majority of these studies focus around the area of animal nutrition. This is due to the unsustainable production of natural feeds (i.e. algae) and rising costs of feedstuffs (i.e. fishmeal

and other protein sources). The Japanese have led the research and development efforts to increase farm yields using formulated diets for over 30 years. A review by Fleming *et al.* (1996) reported the general composition of abalone feed to be 20-50% protein, 30-60% carbohydrate, 1.5-5.3% lipid, and 0.3% fiber. The major component of abalone diets are carbohydrate sources, which parallels their natural diet of algae. Abalone nutritionists not only consider the composition of macronutrients in the diet, but also overall nutritional quality, feed source, feed cost, use of attractants, palatability, digestibility, and form of the diet (i.e. pellet). Diet composition is also influenced by animal size and the differences in metabolism as the digestive system develops through various life stages (Britz & Hecht 1997), as well as temperature, which affects metabolism with respect to protein and energy (Stone *et al.* 2013).

1.1.2.1. Proteins

The majority of operational costs of an abalone farm, like most other types of farms, are derived from feed, power, and marketing of product (i.e. harvesting, packaging, and shipping) (McBride 1998). Of these major costs, farmers can control the feed cost by replacing the most expensive feedstuffs with alternative sources in abalone diets. Protein is the most expensive macronutrient required for nearly all cultured aquatic animals. For most diets, the control or default protein source is fishmeal, which provides the full spectrum of necessary amino acids (AA) in the fish diet. However, fishmeal price has increased over years due to decreases in wild stocks from over-harvesting or climate change events. The use of high levels of fishmeal in diets also contributes to an influx of phosphorus to the natural environment, causing an environmental sustainability issue. The pragmatic solution to this is to use different sources of protein, which could be derived from marine or terrestrial and animal or plant organisms. A study by Uki *et al.*

(1985a) found that casein and soybean meal (SBM) produced the highest relative growth increase for *H. discus hannai* at 56% and 36% BW, respectively, fed at 0.9%/BW/day. Additionally, a study by Guzman & Viana (1998) showed there were no differences between abalone fed a diet with abalone viscera silage and SBM compared to a high fishmeal diet and commercial diet. Although casein is deemed an expensive, impractical alternative to fishmeal, SBM is a less expensive, terrestrial-based source of protein that has been widely used. The benefit of using a plant-based protein source is the high abundance and market availability, and relatively cheap cost compared to fish meal. Defatted SBM is considered as a good alternative source of protein because its AA profile is more comprehensive than other plant-based protein sources. However, it is reported that SMB lacks the proper AA profile and possess anti-nutrients such as protease inhibitors, lectins, phytic acids, saponins, phytoestrogens, antivitamins, and allergens (Francis *et al.* 2001). Despite the removal of hulls using extraction solvents and the heating process that denatures protease inhibitors, some anti-nutrients remains stable in the fed SBM. This means that SBM inclusion levels should be further studied, as well as tested for any allergenic properties it may have on abalone for proper dietary use.

1.1.2.2. Amino Acids

Allen and Kilgore (1975) reported the amino acid requirements of red abalone (*Haliotis rufescens*) after injecting juvenile specimens with radiolabeled glucose. The radioactivity of individual AAs were measured, and the results indicated that nonessential AAs were glutamic acid, aspartic acid, alanine, half-cystine, glycine, serine, and proline. They reported threonine, valine methionine, isoleucine, leucine, phenylalanine, tryptophan, lysine, histidine, and arginine to be essential. This was an important first investigation towards defining AA requirements of

abalone, but this information has its limitations. The authors were unable to determine essentiality of tyrosine, which could play an important role in the protein utilization of abalone. The application of this study is also constrained by the fact that there aren't replicate studies to support or refine these findings, and this particular species of abalone and its gut morphology would potentially differ from other species of cultured abalone. As previously noted, AA profiles have not been investigated extensively, however, it will be important to define which AA are limiting for future commercial diet formulation purposes. If AA profiles are not balanced correctly, the utilization of a given protein source may be negatively affected. Nutritionists must consider that AAs absorbed freely and excessively may be deaminated and this reduces the protein efficiency ratio (weight gain/protein intake) and feed cost; and the opposite condition may also negatively affect growth performance if essential AAs are not supplied, possibly leading to high incidence of mortality. To formulate a diet with a balanced AA profile, nutritionists can use the limited AA of soft tissue analyzed in the laboratory as the reference value for all other AAs. This method reduces the chances of AAs levels being deficient (although some could be in excess, it is better to cover minimal requirements).

1.1.2.3. Carbohydrates

The dietary carbohydrate levels of wild abalone are approximately 40-50% of total intake, which is reflected in the algae they consume. Based on these ecological and feeding behaviors of wild abalone, animal nutritionists have implemented high dietary inclusion levels of carbohydrates at 30-60% in formulated feeds. Today, carbohydrate sources are fairly inexpensive and represent the main dietary component of all formulated abalone feeds. The sources of carbohydrate are derived from terrestrial plants, specifically cereals, which produce starch and gluten. These

cereal-based feedstuffs include but are not limited to wheat flour, corn flour, and rice starch. Starch serves in feeds both nutritionally by adding carbohydrate and energy value and acts as a binding agent to prevent dissociation and leaching of the feed nutrients in the aquatic environment (Fleming *et al.* 1996).

1.1.2.4. Energy

Energy consumption for abalone can differ due to age, size, species, and environmental parameters. Moran and Manahan (2003) reported abalone larvae (*Haliotis fulgens*) to have a 50% decrease in triglycerides in their soft body tissue from 0 to 8 days post hatch, and suggested that lipids were the primary source of energy. In a later study, Viana *et al.* (2007) reported that the same species of abalone larvae (*H. fulgens*) may utilize protein as a preferred energy source during their lecithotrophic larval stage. The authors of this study, found that the relative lipid content increased from 15% to 30% from 0 to 10 days post hatch. However, in the post-metamorphose larvae, the lipid content rapidly decreased to 2% soft body tissue, which reflects a shift in physiological growth and nutrient demand through later stages of development. In both studies, lipid and protein were the predominant sources of energy for abalone larvae.

In contrast, carbohydrate is the primary source of energy for post-settled abalone in culture. Carefoot *et al.* (1993) found that starving Northern abalone (*H. kamtschatkana*) resulted in depleted glycogen reserves in the digestive gland after 6 days. The blood glucose also dropped to nearly half the control level by 6 days of starvation. In a separate study, Beltran *et al.* (2004) found that when abalone were starved, lipids were not mobilized for energy production until at least 70 days of starvation. They reported that animals were using carbohydrates (glycogen) and

then protein as initial sources of energy. Also, the digestive enzyme activities of agarases, carrageenases, celluloses, alginate lyases, laminarinases, amylases, maltases, sucrosases, and beta-galactosidases are indicative that carbohydrates are dietary preference for abalone. According to Fleming (1991), 25 g *H. rubra* has a basal energy requirement of 0.2-0.3 KJ/day. These low metabolic rates suggest that abalone do not require much energy input from their diet. And although excess energy from carbohydrate sources will most likely be stored as glycogen in the muscular foot (which is preferred for taste), overfeeding can result in indigestion of protein, lipids, and other nutrients, and lead to poor feed utilization (Fleming *et al.* 1996).

1.1.2.5. Lipids

This category of nutrient is arguably the most important for aquaculture nutrition in general, and can be applied to the culture of abalone. Lipids provide abalone with fat soluble vitamins, energy, and the essential fatty acids that are critical for cellular support, cellular functions, and reproduction. Lipids are sourced predominantly from fish oil (i.e. menhaden oil, cod liver oil) or from vegetable oil (i.e. canola oil, corn oil, soy oil). Sometimes, however, oils are not added to the feeds and therefore total lipids may come naturally bound to fishmeal. When adding fish oil, it is important to add vitamin E, a natural antioxidant, to prevent rancidity in the feed. Although lipids can be expensive, they constitute a relatively small portion of the total feed. It has been reported that abalone growth rate will be highest at 3.6% and 5.0% lipid levels by Uki *et al.* (1985b) and Bautista-Teruel *et al.* (2011), respectively. The difference between optimal inclusion levels may reflect feed formula differences and the different species used in each study (*H. asinine* and *H. discus hannai*). Higher inclusion of lipids has been shown to detract from overall growth performance. Britz and Hecht (1997) conducted a growth performance and body

composition study on juvenile *H. midae* with three different levels of dietary lipid (2, 6, and 10%). They found that the animals fed with a 10% lipid diet resulted in a decrease in feed conversion ratio. Van Barneveled *et al.* (1998) also reported that lipid content greater than 5% in diet negatively influence AA digestibility and growth performance of abalone *Haliotis laevigata*.

1.1.2.6. Fatty Acids

As mentioned above, lipid inclusion levels greater than 5% in feed results in poor growth performances, however, the relative proportion of fatty acids derived from lipid sources may play an even greater role in the animal's metabolism. Uki *et al.* (1986) suggested incorporating n-3 fatty acids at 1% of the total lipid content of diet and n-6 fatty acids at 3% of the total lipid content of diet. For nutritional purposes, essential fatty acids are defined as fatty acids the abalone can not synthesize *de novo*. Absence of these in the diet leads to deficiency effects. Additionally, polyunsaturated fatty acids (PUFAs), which have greater than two double bonds in the fatty acid structure, account for a third of all fatty acids and are key for animal growth (Tocher 2013). Tocher (2010) found that essential fatty acids (EFA) requirements of aquatic organisms are satisfied by PUFAs 18:2n-6 and 18:3n-3 if they are using fatty acyl desaturases and elongases converting PUFAs to highly unsaturated fatty acids (HUFAs). Despite most fish and invertebrates lacking these enzymes, invertebrates like abalone contain them, which makes them efficient utilizers of fatty acids. According to Bautista *et al.* (2011), the inclusion of EFAs into tissue increased up to the highest dietary level 3.6% tested on *H. discus hannai*, which suggests that 18:2n-6 (linoleic acid) and 18:3n-3 (α-linolenic acid) and n-3 fatty acids are essential for abalone. They also found that this species of abalone can convert or synthesize 20:4n-6 (arachidonic acid) from precursor EFAs supplemented in their diets. They also infer that

low levels of docosahexaenoic acid (DHA) in their samples reflect that DHA is not essential. In another recent study by Mulvarvey *et al.* (2015), abalone fed a macro algae diet known to maintain low DHA levels had higher tissue n-3 profiles than abalone fed formulated diets with higher DHA levels. This, despite displaying the complexity of fatty acid utilization, highlights the importance of fatty acid ratios in abalone feeds. Thus, it can also be said that n-6 or other fatty acids in exceedingly high amounts could reduce the overall conversion or metabolism of fatty acids by abalone. Understanding fatty acid utilization is crucial in the aquaculture nutrition of abalone because EFAs can impact the phospholipid matrix of membranes, influence growth and development, and are precursor molecules for prostaglandins and eicosanoids that affect reproduction (Ishikawa 2007). Industry members will be interested to have market abalone which maintain the proper quantities and appropriate ratios of fatty acids that are known to have human health benefit (i.e. omega-3 and omega-6 fatty acids).

1.1.2.7. Vitamins and Minerals

The vitamin and mineral requirement for abalone have largely followed trout and salmon requirements. Uki and Watanabe (1992) conducted a growth performance trial including different levels of a standard fish vitamin premix in abalone diets. They found that the vitamin premix incorporated into the abalone diets at 8% resulted in highest growth, however, they recommend using around 4% vitamin premix to maintain the appropriate feed quality (good feed solubility). In addition to this baseline understanding of vitamins, the review by Fleming *et al.* (1996) stated vitamin C is especially important and noted abalone lack the ability to synthesize vitamin C *de novo*. Mai (1998) investigated vitamin C and its role in abalone nutrition for two species of abalone. This study showed that there was no significant effect of vitamin C at any

inclusion level on growth or survival of either *H. tuberculate* or *H. discus hannai*. In fact, the control group fed red algae showed no signs of deficiency in vitamin C in their tissue comparatively. Currently, the requirement of vitamin C for abalone remains inconclusive and more studies must be done to refine our understanding. For mineral requirements of abalone, there is minimal information available in the literature. Requirements of dietary zinc for *H. discus hannai* were reported as 16-18 mg/kg from zinc methionine and 35 mg/kg from zinc sulfate depending on the zinc source (Tan and Mai 2001). Dietary selenium requirements were studied and estimated to be 1.41 mg/kg, with toxic effects in levels greater than 9.16 mg/kg (Wang *et al.* 2012). These studies are limited to each species and findings remain preliminary.

1.1.2.8. Attractants and Palatability

When cultured animals are not eating an offered feed, it often suggests that they are not attracted to the feed. Aside from the nutritional aspect of feed formulation, the use of particular feed stimulants or attractants can increase feed intake and improve growth performance of abalone. Each animal uses different sensory mechanisms to detect prey in the wild or feed in culture systems, thus nutritionists consider adding known attractants to their diets.

In a study by Allen *et al.* (2006), *H. iris* was found to spend >80% of their time engaging in feeding-related activities in the presence of macro algae *Gracilaria spp.*, which contrasted the control group (without algal stimulants). This difference in feeding behavior also coincided with a noticeable increase in shell growth of 110 µm/day compared to the control group that averaged 86 µm/day. Although there was improvement in growth rates, not every attractant that increases feed response is palatable (bite or no bite). In a separate study by Viana *et al.* (1994), the authors

wanted to investigate the potential application of 9 different feed stimulants as alternatives to kelp. What they found was that all fish silage treatments (neutralized abalone viscera silage, neutralized fish silage, and acid fish silage) resulted in the highest attractant activity responses due to large amounts of soluble protein and free AAs. However, silage treatments resulted in the lowest palatability, whereas the meals (SBM, fish meal, corn meal, and kelp meal), gelatin, and fresh kelp were highly palatable. In this case, the authors recommended using both combinations of attractant and palatable feed components to achieve a diet with good acceptability. Overall, Fleming *et al.* (1996) reported that attractants are most effective when they maintain specific proteins and/or related compounds (i.e. AAs and peptides). In addition, a novel approach to use all spice as a feed attractant has been investigated and shown to have great potential (Harada *et al.* 1996); however, application in a practical, formulated diet must be done to determine the efficacy of any spice as a feed attractant and stimulant. In abalone aquaculture, the most practical way to increase intake without compromising nutritional density of the feed is to use attractants that increase palatability. An inexpensive option to incorporating feed attractants and stimulants is to use locally sourced or naturally growing macroalgae in powder or meal form.

1.1.2.9. Probiotics

The use of probiotic and prebiotic as feed supplements to enhance performance is a promising aspect for abalone aquaculture. Probiotics not only increase digestibility but also absorption of nutrients by the animal. It has been reported that growth rate of *H. midae* increased by 30% using diets supplemented with probiotics (Macey & Coyne 2005). There are various types of enzyme secreting bacteria that have already been successfully used as probiotic supplements. Doeschate and Coyne (2008) supplemented a kelp-based diet with *Pseudoalteromonas* (C4 strain) bacteria

and observed both an increase in alginate lyase activity in the crop and higher growth rates of *H. midae*. In another study, Rohyati (2015) found various *Vibrio* strains increase agarase activity in the digestive tract of *H. asinine*, which promotes catabolism of monosaccharides and synthesis of non-essential fatty acids. The author observed an increase of over 50% in biomass of the probiotic-supplemented treatment relative to the control treatment in addition to higher feed conversion. Probiotics may also serve to boost the immune health of abalone by competing against harmful bacteria, preventing them from colonizing the abalone gastrointestinal tract, as well as improve antioxidant system response. Research on feed supplements to improve health responses are very scarce, but use of dietary selenium (Wang *et al.* 2012), dietary zinc (Wu *et al.* 2011), and even spices (Fleming *et al.* 1996) have been shown to improve functioning of antioxidant enzymes. In the near future, expanding knowledge of gut microbiology of abalone will serve to benefit aquaculture because the current strains of isolated and culturable bacteria from abalone digestive tracts are limited.

1.1.3. Discussion and Summary

The land-based, high density culture of abalone has been increasing since the decline of wild stocks. The largest aquaculture producing nations are heavily reliant on economies of scale to make operational costs favorable. These costs include but are not limited to energy, labor, and feeds. In terms of feasibility, management needs to consider refining feed formulation because energy and labor are typically fixed to some degree. The use of alternative feedstuffs to offset fixed costs is a key step towards maintaining high yields at a reasonable price point. For farmed abalone sold at $15-35/kg in the US market, using local carbohydrate sources may be the most critical step in reducing operational costs.

Carbohydrates of various sources have been utilized well by abalone due to an active supply of enzymes in the gut of abalone (Fleming *et al.* 1996). Carbohydrates can also offset protein source amounts, which are the most expensive feed ingredients. Protein, although required, has a very large acceptance range of 30-60% inclusion levels; and although substitute sources of protein could reduce the price of traditional fishmeal-based protein diets, defatted SBM and casein have been found to reduce growth performance if substitution is at 100% because of their incomplete AA profiles or presence of growth retardants. Thus, the use of mixed protein sources will be key for developing the optimal diet. More studies are required to determine the exact inclusion ratio of individual protein sources that would support the highest growth rate in abalone. Also, if reducing feed cost is the main priority in nutritional research, investigating protein to energy ratio for each newly formulated diet will be an important aspect to consider. A study by Gomez-Montes *et al.* (2003) showed that growth of juvenile *H. rufescens* was significantly better when offered a diet containing a protein to energy ratio of 100 mg protein/kcal compared to 62, 74, 85, and 108 mg protein/kcal diets. The authors concluded there to be a potential reduction in dietary protein and lipid levels without adverse effects on growth.

Although lipids have been found to decrease growth performance in abalone at inclusion levels exceeding 5%, oils high in 18:2n-6 (linoleic acid) and 18:3n-3 (α-linolenic acid) in combination with vitamin E (reduce rancidity during storage) can be included to promote absorption of fat soluble vitamins and improve cellular structure and function. The inclusion of proper fatty acids would also potentially improve reproductive success and egg and larval quality, which would be vital for abalone hatcheries.

For attractants and palatability, the use of fish meal or fish by-product combined with a macro algae meal should be used into the formulated diet. The rate of feeding can outweigh nutritional

content of feed in circumstances where animals are not feeding. Formulating a diet that attracts the animal and maintains palatability is crucial for mollusk aquaculture, and using by-products and local sources of macro algae are relatively cheap yet maintain good nutritional content.

For vitamins and minerals, use of a standard vitamin premix for fish can be incorporated at 4-8% inclusion levels. Despite vitamin C, zinc, and selenium being studied for their roles in abalone nutrition and health, other individual vitamins and minerals appear nutritionally adequate.

The use of probiotics and prebiotics are an upcoming field with immense potential to increase growth performance and health in abalone. However, more studies are required to determine which particular probiotic and prebiotic at which dose level will work most effectively for a particular diet.

Overall, abalone as an aquaculture species is the perfect case-study mollusk for related invertebrates. Despite the variety of different species from the genus *Haliotis*, many have been cultured routinely using formulated feeds and land-based systems in various regions of the globe. The current supply of farmed abalone outweighs wild harvested abalone, which is remarkable considering there is still so much research that can be done to improve aquaculture production.

1.2. Limpet

The current research on aquaculture of limpets is far behind abalone regarding the development of technologies to produce captive bred animals. Despite this lack of aquaculture research, the ecology of limpets is well understood. The laboratory system should therefore reflect what we know about limpets living in their natural environment. Additionally, the culture of abalone and

other commercially important mollusks could be considered as model species with proven technologies in aquaculture to advance the production of limpets.

In terms of ecology, limpets are easy to study as they reside in the nearshore environment and are easily accessible to humans. Like abalone, limpets are herbivorous grazers that feed on benthic diatoms and microalgae growing on rocky substrates. The teeth on their radula have been found to exhibit the highest tensile strength out of any biologically produced substance, which allows them to graze on the toughest crustose coralline algae with ease (Barber *et al.* 2015). In terms of their evolution, these marine snails are polyphyletic, suggesting their biology have developed through various independent lineages to derive at groups of animals called limpets. These evolutionarily convergent limpets, all defined morphologically by their conical shape, fall under various clades (i.e. *Vetigastropoda*, *Neritimorpha*, and *Heterobranchia*). The group of true limpets is classified under the family *Patellidae*, which is the major constituent group of the larger entity deemed limpets. Limpets have a wide geographic distribution that spans from the tropics to the poles. Depending on the species, limpets have been found to inhabit the upper limits of the littoral zone to sublittoral zone, largely influenced by environment (i.e. desiccation) and biology (i.e. competition and predation). As for reproduction, sexual maturity is observed to range from just 6 months to a year, depending on the environmental and biological pressure. They maintain spawning periods around the time of year that is most conducive for successful recruiting; and it has been hypothesized that wave, lunar cycles, and temperature influence gametogenesis. Healthy populations of limpets can maintain high densities in the wild because they broadcast spawn, maintain high fecundity, and are fast growing; all attributes of a candidate aquaculture species (Hua & Ako 2012).

Limpets, although resilient to environmental and biological pressure from other invertebrates, have been subject to years of negative anthropogenic influences. The causes of population declines in coastal regions of North America and Hawai'i are overharvesting, fishing, and habitat fragmentation. Archaeological influences show that over 12,000 years of human influence on *Lottia gigantea* off the California coast has resulted in decrease in the maximum size, mean size, and overall abundance (Erlandson *et al.* 2011). In Hawai'i, a study on the history of the three endemic limpet species (*Cellana exarata*, *C. sandwicensis*, and *C. talcosa*) revealed that limpet populations have largely been depleted in the main Hawaiian Islands due to over harvesting. At the turn of the 20th century, total catch landings were nearly 68,039 kg $year^{-1}$ and in 2008 total catch landings drastically decreased to 4,763 kg $year^{-1}$ (Kay & Magruder, 1977; Tom 2011). Although the degree of human influence may vary by geographic region, the striking similarities of the relationship between limpets and humans persist today. For both Native Americans and Native Hawaiians, limpets were not just seen as a good source of protein, vitamin A, vitamin D, phosphorus, and iron (Miller and Robins, 1940), but also a cultural symbol. They used these animals and their shells as scraping and scaling tools, drinking bowls, and medicine; and in Hawai'i, certain families revere the opihi as their family god or aumakua (Tom 2011). The price of opihi was reported to be around US$10 kg^{-1} in fish markets, which reflects the importance of this local seafood (Thompson 2011).

As with most significant fisheries around the world, continual decline in wild populations of limpets has implications not only ecologically, but also socioeconomically and culturally. The growing demand for this high priced seafood beckons for aquaculture to offset the scale. Aquaculture of limpets, although extremely limited, is currently being developed with hopes to

supply the market with sustainably sourced seafood, improve wild stocks, and produce key medicines (Gibson 1978; Corpuz 1981; Harris & Markl, 1999; Hua 2014).

1.2.1. Culture Systems

Culture system technology for farming various species of limpets have been sporadically developed and, by default, follow the protocols of mollusk aquaculture in general. Many of the limpets used in aquaculture have only been studied for one life stage (i.e. juvenile nutrition trials, broodstock spawning trials) with few species' life cycles closed. The fact that there are missing pieces to the puzzle in culturing a species from larvae to successful adult reproduction results in a deficiency in limpet farming protocols available for commercial scale operation.

The culture system design for limpet aquaculture addresses two major concerns: their ability to firmly attach to substrate and their sensitive feeding behaviors. When limpets suction to the substrate, moving them from tank to tank poses mortality risks (up to 50%), and their preference for vertical surfaces, slow grazing movements, and nocturnal feeding tendency makes feeding them more difficult to culture than the active abalone (Hua 2014). To cope with these mechanistic issues, Hua (2014) first used polyethylene liner to the tank that allowed for production of natural diet (biofilm) as well as easy removal of animals from their substrate. In formulated-diet-based growth performance trials, Hua designed removable, suspended holding containers constructed of plastic mesh lining (cone and cylinder shaped), which made identification, handling, and feeding individuals much easier. These cone and cylinder shaped containers not only increased vertical surface area, but also allowed for semi-moist feed to be adhered to the substrate for feeding official diets. Overall, the use of polyethylene sheets

appeared to work best when offering natural, biofilm diets and suspended holding containers worked best for implementation of formulated diets. In the future, for commercial purposes, limpets that don't need to be forcefully removed from their substrate would foreseeably do well in standard mollusk culture systems (i.e. abalone cages, baskets, or plating systems). This choice in system design, however, is subject to what feeds would be made available (natural diet vs. formulated diet), the density at which limpets would be farmed and the environmental parameters required (i.e. spray or mist for limpet species residing above the water).

1.2.2. Spawning Technology

Although the spawning of abalone has become routine, the sustainable aquaculture of limpets will depend on successful spawning and larval rearing. For this paper, it is necessary to discuss the methodology of spawning limpets to better understand what current limitations there are. The improvements of farming techniques for limpets have primarily been focused on spawning and reproduction, predominantly suiting larval development studies as opposed to food production. Many of these developmental biology studies utilize aquaculture and laboratory controlled spawning events because it is difficult to study in wild limpet larvae. Researchers collected wild, mature animals during the observed spawning season for a given species, and kept the animals in flow through systems until they induced spawning (Kay & Emlet 2002). In Hawai'i, locals believe the spawning periods are linked to the lunar cycle, and collection of wild animals for spawning is suggested to be optimal around the new moon (Tom 2011). But spawning healthy limpets naturally in laboratory conditions is shown to be difficult, and use of alkaline solutions, vigorous aeration, thermal shock, desiccation, and artificial insemination are used alternatively—

both independently or in combination (Corpuz 1981; Kay & Emlet 2002; Perez *et al.* 2007; Reynoso-Granado *et al.* 2007; Aquino De Souza *et al.* 2009).

Spawning of limpets is important, but also difficult and often the limiting procedure for scientists studying developmental biology or farmers intending to produce juvenile animals for market. Spawning events and larval rearing are inherently difficult because they require controlling for environmental and biological parameters during the animals' most dynamic and vulnerable life-stage. For the purpose of this paper, it should be noted that closing the life cycle of specific limpets is dependent on this still unrefined dimension of aquaculture.

Based on the literature, *Lottia digitales*, *Lottia. asmi*, *Fissurella volcano*, *Patella depressa*, *Patella vulgata*, *Cellana exarata*, and *Cellana sandwiciensis* have spawned in captivity with limited success (Corpuz 1981; Kay & Emlet 2002; Reynoso-Granado *et al.* 2007; De Souza et al, 2009; Hua 2014). In fact, the only documented spontaneous spawning of limpets in captivity was by Reynoso-Granados *et al.* (2007). In this study, *F. volcano* were offered microalgae (*Nannochloropsis spp.* and *Phaeodactylum spp.*) over the course of 40 days until spawning occurred naturally. The interesting aspect of this study was that the authors chose to offer *Nannochloropsis* and *Phaeodactylum*, neither of which occur in the natural environment of *F. volcano.*

A common method for spawning invertebrates is subjecting mature animals to hydrogen peroxide (H_2O_2) solutions. Kay and Emlet (2002) adopted this method to successfully spawn *L. digitales* and *L. asni* in captivity. The release of hydroperoxy free radicals (HOO-) and peroxy radicals (OO-) from H_2O_2 interacting with seawater is believed to activate prostaglandin endoperoxide synthetase and induce spawning (Morse 1984). But this is not a sustainable practice for the limpet aquaculture industry because it requires capture of mature animals.

Hydrogen peroxide was also observed to have lethal effects on broodstock opihi, which makes it unsuitable for implementation (Hua 2014).

Another method used is artificial fertilization by removal of gametes and induction of oocyte maturation in NaOH-alkaline seawater. This method was first used by Corpuz (1981) and second by De Souza *et al.* (2009) to successfully observe larval development of *C. exarata*, *L. digitales*, and *L. asni*. Again, this practice is unreasonable for the limpet aquaculture industry because it requires an intricate and lengthy process to derive larvae.

The most practical method for spawning limpets appears to be injecting Gonadotropin Releasing Hormone (GnRH) directly into the animal's gonad. In a study by Hua and Ako (2012), eight wild opihi were collected during the natural spawning period (assumed to be sexually mature) and each animal was injected with a single high dose (1,000 ng/g BW) of GnRH. The resulting spawns yielded larvae that were grown to 9 days post hatch (DPH) on various inoculated biofilms. Despite unsuccessful rearing of opihi, this method did not result in high mortality of broodstock animals compared to experiments using hydrogen peroxide.

Once broodstock limpets undergo spawning, the eggs and sperm are collected separately and carefully fertilized with minimal or no aeration in small hatching containers. When the fertilized eggs develop over 12-24 hours, the larvae are collected and rinsed. Larval systems for limpets follow the lead of sea urchin and abalone larval systems. The optimal set-up being a conical tank with moderate, central aeration allowing for good circulation within the tank to promote proper water simulation. These larvae are offered cultured microalgae in their pelagic larval stage, lasting for a few days to a few weeks depending on the species. Once animals are considered competent, they are transferred to a nursery system for settlement and metamorphosis.

It has been found that limpets prefer vertical surfaces to settle on, so glass or plastic settlement plates designed to increased vertical surface area are placed in the nursery system (Corpuz 1981; Hua 2014). These settlement plates are also inoculated with microalgae and exposed to a light source to produce a biofilm that serve as the primary food source for developing juvenile limpets. It is therefore critical to choose nutritionally adequate food sources that are also known to induce settlement and metamorphosis- a determinant for survival between larval and juvenile stages. The identification of specific bioactive compounds produced by specific algae and/or bacterial films that cues settlement and metamorphosis of most invertebrates have only been understood on the macroscopic level (Hadfield & Paul 2001).

For two species of opihi, the first attempts to rear larvae by Corpuz (1981) and Hua (2014) resulted in poor settlement and metamorphosis with naturally occurring biofilm. This may be due to the biofilm used, albeit naturally occurring and nutritionally adequate, most likely lacked proper bioactive compounds or there were water quality issues. Natural opihi habitat is dominated by crustose coralline algae (CCA) (Tom 2011). Despite the knowledge gap in species specific interactions regarding settlement cues, CCA has been the most thoroughly investigated algae in its role in inducing settlement and metamorphosis for various invertebrate species. The cell wall-associated compounds, namely glycoglycerolipids and polysaccharides, were found to successfully induce settlement and metamorphosis of coral larvae (Tebben *et al.* 2015). Crustose coralline algae was also found to significantly increase settlement for the fluted giant clam Tridacna squamosal, which highlights the potential implementation for other invertebrate larvae such as opihi (Neo *et al.* 2008). However, the long-term feasibility of growing crustose coralline algae for larval rearing is yet to be determined, and alternative methods should also be explored.

1.2.3. Nutrition and Formulated Feeds

There is no surprise that preliminary limpet aquaculture studies have used biofilm to grow animals. A bioenergetics study by Niu *et al.* (1998) used biofilm to determine the annual energy consumption of *Lottia kogamogai*, which resulted in 0.20, 1.42, 3.06, 4.75, 5.85 and 6.97 kJ $year^{-1}$ $individual^{-1}$ for groups ranging from 0 to 5 years, respectively. Microalgae contain both energy and essential fatty acids in high concentration. They also provide growth promoters (i.e. phytonutrients) and are good attractants. The various microalgae in aquaculture have been studied and analyzed for their contribution towards growth of various species of invertebrates; and of the commonly used species in mariculture (*Chaetoceros calcitrans, C. gracilis* 1, *C. graciis* 2, *Skeletonema costatum, Thalassiosira pseudonana, Isochrysis* spp., *Pavlova lutheri, Nannochlois atomus, Tetraselmis suecica* 1, *T. suecica* 2, *Choomona salina* 1, *C. salina* 2), diatoms *Chaetocerous spp*. maintain significantly greater amounts of n-3 fatty acids (Volkman et al., 1989). Hua (2014) analyzed the gut content of opihi microbiota and reported that the dominant species were *Bacillaria paxillifer, Fragilaria spp*., *Melosira spp*., *Navicula spp*., and *Rhabdonema spp*.; in average abundance were *Amphora spp., Climacosphenia spp*., *Licomophora spp*., *Nitzchia spp*., *Pleurosigma spp*., *Tabellaria spp*., and *Trigonium spp*.; and other species identified were *Asterionella spp*., *Cymbella spp*., *Diplonesis spp*., *Masogloia spp*., *Opephora spp*., *Surirella spp*., and *Thalassionella spp*.. Although limpets consume a diet consisting of microalgae, both microalgae and macroalgae complement each other and have been considered as stand-alone feed or feed additives for various life stages of invertebrate aquaculture. Daume (2006) investigated the roles of bacteria, micro and macroalgae in abalone aquaculture and reported that the industry is dependent upon cultured algae to for larval rearing. Microalgae and their associated bacteria were found to play key roles in larval settlement and

inducing metamorphosis; and this can be said for sea urchin larval rearing as well (Daume 2006). Daume (2006) also stated that implementation of macro algae (Ulva and Macrocystis) also promote healthy larval rearing as a substitute for crustose coralline algae - the preferred food. The preference of crustose coralline algae becomes problematic due to the unsustainable nature of culturing crustose coralline algae in tank systems (Daume 2006). The need to digress from using crustose coralline algae for settlement and diet opens up discussion for implementing different compositional biofilms and formulated diets. If we look at abalone and sea urchin aquaculture, macro algae has potential use as suitable natural feeds in commercial production of juveniles (Viera *et al.* 2005). Similar to microalgae, culturing macroalgae has proven to be largely unsustainable; additionally, limpets do not readily feed on clumps of macroalgae as abalone and sea urchins will, henceforth diets for limpets should use macroalgae meals as supplements to a basal diet.

Currently, nutritional requirements of limpets are largely unknown and we have, up until recently, made baseline assumptions from what we already know about other mollusks. Like abalone and sea urchins, the primary source of energy for limpets is carbohydrate as supported by examination of their natural food sources. The naturally growing benthic crustose coralline algae, microalgae, and diatoms that are grazed upon by limpets maintain nutritional profiles suitable to sustain limpets in a laboratory environment (Oakes *et al.* 2004). But inoculating and culturing biofilm for long-term grow-out periods has been shown to be unsustainable (Hua 2014). The use of formulated feeds should be seen as a requirement for further developing limpet culture; and for the purpose of this paper, the dissertation work of Hua (2014) has been used as a case study for derivation of the current formulated opihi feed.

Hua (2014) conducted a series of feeding trials to determine the proper feed ingredients for *C. sandwicensis*. Major feed ingredients such as green tea, silk worm pupae, casein, fishmeal, SBM, krill meal, and corn meal; all of which were used previously in abalone growth performance studies were considered (Cho 2008; Cho 2010). The first trial, which was based on convenience, used previously formulated commercial sea urchin diets. Of the different diets offered, the ones containing fishmeal and SBM were preferred, however, it should be noted that there was poor palatability for all sea urchin diets. After selecting the base ingredients as fishmeal and SBM, the investigator looked into improving palatability of the feed. In the same preliminary trial, the author used fresh biofilm as an attractant in comparison to spirulina, gamma-aminobutyric acid (GABA) and dimethyl propiothetin (DMPT). As a result of the subsequent diets offered, opihi performed best on a fishmeal/SBM/biofilm diet. In a following trial, the author then used various commercial macroalgae (Norwegian kelp *Asophyllum nodoscum*; "ogo" *Gracilaria pacifica*; "kombu" *Laminaria japonica*; "nori" *Porphyra spp*. Nishimoto Tradng Co. Ltd., Korea), as a possible replacement for biofilm used as an attractant. Nori or prepared red algae from the genus *Porphyra* was found to be the best attractant, and this was considered to be a key finding in formulating a sustainable diet and was used in diets of all subsequent studies. Following the palatability trial, Hua (2014) proposed using fishmeal and SBM in combination with krill meal or corn meal. The results of this trial indicated that opihi performed significantly better on fish/SBM/krill/Porphyra (0.73% DM/BW/day) in comparison to fish/SBM/corn/*Porphyra*, fish/*Porphyra*, and biofilm (control). Finally, in a separate trial, Hua (2014) addressed the issue that opihi are slow grazers and like to feed on vertical surfaces. To feed opihi efficiently, the diet must maintain good water stability and be semi-moist for application to the walls of the tank surface. Results from this trial showed that gelatin appeared to hinder feeding, and agar did not

pass stability testing; instead, alginate at 6% worked well to create the proper textured feed. From the combined results of these first set of feeding trials, it can be concluded that a suitable first basal diet should mainly consist of fishmeal, SBM, krill meal, *Porphyra* as an attractant, and alginate as a binder.

1.2.3.1. Proteins

For the majority of cultured animals, determining the protein requirements is a key first step in formulating a sustainable feed. In terms of limpet protein requirements, a nutritionist can attempt to match the AA profile of the diet offered to that of soft tissue samples from the target species with diet offered. Despite utilization of nutrients varies from species to species and animal to animal, this concept is one way nutritionists have managed to approximate values of unknown dietary requirements in the formulation of an optimal diet (Fleming *et al.* 1996; Hua 2014).

Hua (2014) conducted the first protein requirement study around this AA-matching principal by using a basal diet of fish/SBM/krill offered at protein levels ranging from 21% to 50%. To offset decreases in protein, diatomaceous earth and starch were used to balance the diets. The results of the study showed that opihi growth rate does not increase as the dietary protein increases. In fact, there was no significant ($P<0.05$) difference between 27% and 47% protein diets and use of polynomial analysis of insignificant differences revealed that 35% protein was the optimal dietary level. It was noted by the author that although the AA profiles of the diets matched the soft tissue of opihi, other factors such as feed consumption rate also plays an important role. The animals fed the highest level of protein (50%) maintained low feed consumption, which correlated with decreased growth. Although more studies are required to support and refine the

protein requirements of opihi, particularly gross energy consumption, a 35% protein inclusion level lies within the range of abalone protein requirements (Fleming *et al.* 1996). The energy requirements should also be considered to insure proper utilization of protein at given levels.

1.2.3.2. Carbohydrates

Carbohydrate utilization is often determined by the gut microflora of marine organisms. According to Hua (2014), assumptions can be made that limpets should efficiently use high dietary levels of carbohydrate sources because high amylase activity in the gastrointestinal tract of *Patellacean* limpets has been observed in various studies. An abalone study by Lee *et al.* (1998) evaluated the dietary carbohydrate sources (wheat flour, dextrin, sucrose, and potato starch) for abalone and the results revealed that weight gain was not significantly different than control diets (Laminaria and Undaria). This suggests that carbohydrates of various sources could be used for limpet aquaculture feed. The studies by Cho (2008) and Cho (2010) also show that alginate can be used in abalone diets, however, alginate is expensive and should be limited to use as a binder or eliminated entirely.

As for the baseline carbohydrate study conducted by Hua (2014), opihi were fed four different diets with carbohydrate inclusion levels ranging from 18% to 37%. The aforementioned basal diet was used for this study, maintaining the protein and lipid levels at 26.5% and 3.5%, respectively. The results showed that there was no significant difference in specific growth rate between 18% and 32% carbohydrate levels, however, the polynomial regression shows optimal dietary carbohydrate to range from 27% to 32%. Additionally, if economic viability of feeds is a priority, it is suggested that 32% carbohydrate be used to offset protein cost. Hua also noted high

mean feed conversion rate (FCR), which indicates that opihi are efficient converters of feed to body weight gain.

1.2.3.3.Lipids

Although crude lipid requirements are currently unavailable, researchers have assumed dietary lipids are not as important for growth performance as other macronutrients. If limpets do maintain similar dietary requirements, which in many regards it appears that way, lipid inclusion levels greater than 5% could pose health risks (Fleming *et al.* 1996). This is due to low or negligible lipase activity compared to other digestive enzymes in the gastrointestinal tract of abalone; and the composition of these enzymes allow for efficient conversion of feed to body weight from protein or carbohydrate sources in contrast to lipids sources.

1.2.3.4. Fatty Acids

Assuming that limpets are similar to abalone with regards to lipids, specific fatty acids contained in lipids of could play an essential role in other biological processes such as gametogenesis and sexual reproduction. A recent study by Gesto *et al*. (2016) evaluated the role of retinoid in limpet *Patella vulgata* by quantifying the expression of retinoic acid receptor (RAR) and retinoid X receptor (RXR) in wild samples. The authors found that these vitamin A derivatives—known to aid in meiosis—were expressed higher in the gonad tissue of breeding individuals, which supports that retinoic acid maintains a role in gonad maturation. This finding opens discussion for use of particular fatty acids in formulated diets to induce maturation of limpets. In a previously mentioned study, Hua (2014) formulated a maturation diet using arachidonic acid

(ARA) and eicosapentaenoic acid (EPA) at specific ratios. Arachidonic acid is a precursor biomolecule for prostaglandins that promotes reproductive health. In abalone, essential fatty acids were found to be 18:n-6 and 18:3n-3; and *Haliotis discus hannai* was found capable of converting these to 20:4n-6 (ARA), however, these conversion rates may be slower than desirable for aquaculture (Bautista *et al.* 2011). The supplement of ARA and EPA in the proper ratio has been shown in various marine aquaculture species to induce maturation and improve spawn quality (Tamaru *et al.* 1992).

Hua (2014) referenced the fatty acid composition of the diatoms found in the gut of opihi to determine the range of ARA/EPA ratio values to testing. This led to the development of a final maturation diet, which was composed of the basal diet that was refined twice over by protein and carbohydrate requirement studies in addition to pure arachidonic acid. In terms of the experiment, the aforementioned grow out diet (control) was compared to the low ARA diet (0.20g/100g) and high ARA diet (0.33g/100g), both of which were kept at a ratio of 0.7 ARA/EPA. The results showed that when given the proper final maturation diet, regardless of low or high ARA treatment, the Gonadal Somatic Index (GSI) or measure of maturity was twice that of the control group. It was noted that these data may underrepresent the total effect of the ARA treatments because the opihi were accidentally given diets with an inappropriate level of ARA 0.04g/100g during the first phase of the experiment. Another important potential factor was that the second phase of the experiment that successfully yielded mature animals (based on GSI) coincided with the natural spawning periods of December to January (Kay *et al*. 2005). Replication of final maturation in laboratory environments are required to further determine if ARA offered in diets can consistently produce sexually mature animals, and if the ratio of fatty acids affects spawn quality.

1.2.4. Discussion and Summary

The aquaculture of limpets is relatively undeveloped compared to abalone. Much of the attention for limpet aquaculture is on developmental biology; however, the market demand to expand farming of mollusks has beckoned for limpets to be produced for food. There are many issues in limpet aquaculture because of their sensitive nature and complex environmental and biological requirements, most of which is still unknown in laboratory environments.

What research has been able to explain is that handling of limpets is best using plastics or holding containers to prevent permanent attachment to substrate and to efficiently feed. The land-based system designs have pulled from the established protocols of abalone culture along with much of the baseline nutritional understandings. The critical step in successfully developing limpet aquaculture on a commercial level is inducing gametogenesis and spawning animals in captivity. To do this, the focus in limpet research is largely pertaining to inducing final maturation and investigating how to routinely spawn animals in a way that does not result in mortality of the broodstock.

The use of a basal diet made up of fish meal, SBM, and krill meal was shown to work best for ‘opihi aquaculture (Hua, 2014). The use of macro algae as an attractant was critical for getting ‘opihi to feed; this was a larger issue than having proper nutrients in the feed during some of the preliminary researches conducted by Hua (2014). Although krill meal was shown to increase feed intake, it is not a feasible feed ingredient for commercial application due to its high cost. Thus, replacement of krill meal on a one for one basis with an alternative, terrestrial-based protein source should be tested. The requirements, although based on a single study, should follow as suggested with 35% protein, 32% carbohydrate, and <5% lipid.

As mentioned for abalone, spawn quality is often correlated to diet quality. It was found that diets including ARA/EPA in a 0.7 ratio were capable of inducing final maturation in ‘opihi (Hua & Ako 2012). However, before we can be confident in using formulated diet as the sole methodology for inducing final maturation, the consistency of diet-induced final maturation must reach industry standards.

Many cultured vertebrates and invertebrates have shown their inability to spawn in laboratory or farm environments naturally (Podhorec & Kouril 2009). Hua (2014) discussed the next best alternative maturation induction methodology for limpets, which is using GnRH. The GnRH is a neuropeptide hormone that controls the reproduction in many vertebrates and invertebrates. Many bioactive forms of GnRH were found in invertebrates (i.e. sea hare and abalone) (Nuurai *et al.* 2010). According to Nuurai *et al.* (2010), abalone were found to reach final maturation over a 5-week period via injections of GnRH at a low dosage of 250 ng/g as well as a high dosage of 1,000 ng/g. The GnRH was found to affect the neurosecretory system, ultimately leading to the secretion of hormones from the ganglia, which regulates gametogenesis.

Hua and Ako (2012) conducted a study attempting to induce final maturation in ‘opihi based on the assumption that ‘opihi will maintain similar biological function to that of abalone. ‘Opihi were injected with salmon GnRH analog at 250 ng/g dosages per week against a control group injected with saline water. Over the 6-week period, histological samples were analyzed each week, and a peak GSI of 32.9% was observed at week 4 for animals injected with GnRH. The control group maintained the same GSI throughout the trial, which was indicative that GnRH affects gametogenesis.

Moving forward, we suggest implementation of a maturation diet in combination with 2-4 injections of GnRH at 1000 ng/g to induce spawning of limpets during the natural spawning season. This would most likely yield the highest GSI and the highest quality spawn.

In the event of a high-quality spawn, it can be recommended using a biofilm of specific microalgae commonly found in the gut of adult limpets. The biofilm type would be determined by which microalgae are culturable and available to the researcher. For instance, despite CCA potentially being a key source of food and inducer of settlement for opihi in the wild, CCA is not routinely cultured in land-based systems. For ‘opihi, based on results from the first attempts to rear *Cellana sandwicensis*, it can be recommended to inoculate settlement tanks with a mixture of *Amphora spp*. and *Pavlova spp*. at a minimum of 105 cell/cm² (Hua 2014). Combinations of benthic and pelagic microalgae will be required to settle and metamorphose opihi, and various types should still be examined to determine the best. In addition, the use of conspecifics in settlement tanks may increase settlement and metamorphosis. We suspect this to be the case because specific epibenthic microalgae commonly grow on ‘opihi adults.

Overall, the movement to formulated diets from natural biofilm diets, and routine spawning protocols are the next step in successful commercial aquaculture. Without the sustainable reproduction of broodstock, grow out juvenile limpets will not be deemed feasible. The development of commercially viable diets of limpets will also remain at bay, provided the technology to rear limpets in laboratory settings is not developed. The capture of wild limpets for grow-out studies is not sustainable because populations have been over-harvested and continue to dwindle, globally.

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Chapter 2

Effects of dietary protein to energy ratios on growth performance of yellowfoot limpet (*Cellana sandwicensis* Pease, 1861)

Abstract

The aquaculture of yellowfoot limpets (*Cellana sandwicensis)* is a prospect industry in research and development. The effects of dietary protein to energy (PE) ratio on growth performance were evaluated for 180 days in a flow-through system. Replicate animals (5.9 ± 1.72 g and 33.9 ± 2.13 mm) were stocked randomly on individual plates, and four paste diets containing PE ratios ranging from 87.2 to 102.9 mg/kcal were offered once daily (1600 h). A significant increase in daily feed intake ($p < 0.05$) was observed to coincide with seasonal decrease in air temperature. Although dietary treatment had no significant effect on overall growth performance ($P > 0.05$), average daily gain (ADG) and feed conversion efficiency (FCE) improved both linearly and quadratically (ADG $p = 0.03$, $p = 0.08$; FCE $p = 0.05$, $p = 0.04$, respectively). These results indicate potential seasonal growth patterns, which are controlled by environmental cues (i.e. temperature, feed availability, etc.) and must be considered in future trials. Limpets offered higher PE ratio diets did not compensate for lower energy levels with increased feed intake, and specific growth rate increased up to 0.20 % BW/d as the dietary PE ratio decreased. A PE ratio of 87.2 mg/kcal produced the best tissue growth and can be recommended as a suitable formulated diet for limpet production.

2.1. Introduction

A group of mollusks known as limpets (order *Patellogastropoda*) are important seafood derived from the rocky intertidal environment (Erlandson *et al.* 2011; McCoy 2008). These mollusks are usually wild harvested for food consumption; however, continual exploitation has pushed some populations to the brink of extinction (Espinosa *et al.* 2009). Furthermore, declines in wild stocks have pushed governments to intensify management efforts as well as to consider the development of limpet aquaculture (Mau and Jha, 2017). For instance, in South Africa, the government designated multiple "Marine Protected Areas" to preserve the overharvested South African limpet (*Cymbula oculus*) (Branch and Odendaal 2003). And in Portugal (1993-1998), the Regional Government of the Azores implemented a law to ban the wild harvest of two species of limpets, *Patella aspera* and *P. candei* (Ferraz *et al.* 2001).

In Hawai'i, the native group of limpets, referred to as opihi (*Cellana spp.*), are consumed as a staple food during traditional gatherings. Despite management efforts and law prohibiting harvest of Hawaiian limpets less than 31 mm in shell length (SL), there has been a drastic reduction in market availability. Total annual catch landings of Hawaiian limpets decreased from about 68,000 kg to 5,000 kg since the early 20th century (Kay and Magruder, 1977; Bird 2006); and population densities have decreased by 99.9% for the island of Oahu since western contact (Personal Communication; CE Bird, 2017). To prevent complete decimation and overcome market deficiencies, optimizing a grow-out diet is required to support aquaculture production of these socioeconomically important limpet.

For yellowfoot limpet (*Cellana sandwicensis*), the first formulated diet was developed following formula and dietary guidelines used for abalone feeds in a study by Cho (2010). Later, Hua and Ako (2016) found optimal protein and carbohydrate requirement for adult yellowfoot

limpet to be 35% and 32%, respectively. Based on this study, yellowfoot limpet appears similar to abalone (*Haliotis*) with respect to feeding behaviors, metabolism and nutrition. However, the gross energy levels were not measured and requirements are still unknown.

In other studies, both the South African abalone (*Haliotis midae)* and green abalone (*H. fulgens*) were shown to consume feed based on their energy requirement with respect to dietary protein to energy (PE) ratio, which ranged from 43 to76 mg/kcal (Green *et al.* 2011) and from 62 to 108 mg/kcal (Gomez-Montes *et al.* 2003), respectively. Although direct comparisons cannot be made between different aquaculture groups, these results were similar to that of shrimp (*Penaeus monodon*) where an increase in energy with respect to a constant protein improved growth performance (Bautista, 1986). These findings led to the hypothesis that feeding and growth performance of yellowfoot limpet would be affected by the dietary PE ratio. To best of our knowledge, there is no such information available for yellowfoot limpet. Therefore, the objectives of this study were to develop, fabricate and implement a novel grow-out system, and to evaluate the effect of varying PE ratio diets on growth performance of yellowfoot limpet.

2.2. Materials and Methods

2.2.1. Animal collection

Altogether 90 wild adult yellowfoot limpets were collected from a non-disclosed intertidal location in Puna, Hawai'i. The smallest, legal sized animals (approximately 31 mm SL) were selected and carefully removed using metal putty knifes. Prior to stocking, a standard 45 L cooler was filled with natural seawater, chilled to 15 °C, and supplied with aeration. Upon removal

from the rocks, animals were allowed to adhere themselves to black acrylic plates, which were designed to stand vertically in the cooler. Limpets were transported within 48 h without feeding to the research facility. Upon arrival, animals were transferred into circular tubs supplied with overhead irrigation spray for quarantine and acclimation at ambient, outdoor conditions.

The conditioning of animals to a formulated feed (Hua and Ako, 2016) were initiated weeks prior to the start of the trial. Limpets were fed daily to satiation and showed no signs of acceptability or palatability issues; and animals consumed feed effectively without knocking feed off surfaces.

2.2.2. Diet preparation and analysis

Four experimental diets were formulated (Table 2.1.) to make different PE ratio. To test the effect of PE ratio of diets (Diet1, 87; Diet2; 95; Diet3 97; Diet4, 103) on growth performance of limpet, crude protein was kept constant (40%) and gross energy level was graded (3.85-4.63 kcal/g). Krill meal and *Porphyra* were found to be necessary attractants in the feed and were kept constant. Alginate was used as a binder at 5% in all diets. Diatomaceous earth was used as a filler as whole wheat volume was reduced. Assuming limpets are inefficient users of fat, crude fat was kept constant at 6% for all diets. A vitamin premix (MP Biomedical LLC, Solon, OH) used for previous abalone diets were included at 1% in all diets. All diets were analyzed for their proximate nutrients, amino acid and fatty acid profile (Tables 2.1., 2.2. and 2.3., respectively).

The four diets were moist feeds that were adhered to vertical surfaces by pressing feed to the substrate surface. To make the diets, dry starch ingredients (whole wheat and alginate) were homogenized in a food mixer for 10 minutes. Water was boiled and added in a 1:1 ratio (1 mL

water: 1 g dry ingredient) along with oils to the mixture. Starches were homogenized for an additional 10 min. The rest of the dry ingredients were homogenized and added to the gelatinous mixture and further homogenized until reaching a dough-like consistency. To dry, the dough was rolled out into 1 cm thick sheets and air dried at room-temperature until cooled (approximately 30 min.) and feeds were placed in the freezer until use.

Feed samples were analyzed for proximate composition using methods of AOAC (2006). Moisture content was determined from a 2 g sample using an air-circulated oven at 135 °C for 2 hours (method 930.15) followed by ashing in a muffle furnace at 600°C for 6 hours (method 942.05). Crude protein was estimated by determining total nitrogen (N) by dry combustion using a LECO analyzer (LECO CN-2000; Leco Corp., St. Joseph, MI, USA; method 976.05, CP = N × 6.25). Crude fat (lipid) was determined by ethyl-ether extraction (method 920.39) using an Accelerated Solvent Extractor (Dionex Corporation, Bannockburn, IL). Gross energy (GE) was determined using an oxygen bomb calorimeter (Parr Isoperibol Bomb Calorimeter 6200, Parr Instrument Co., Moline, IL) with benzoic acid as the calibration standard. Minerals were analyzed by inductively coupled plasma atomic emission spectroscopy (Thermo Jarrel Ash Corporation, Franlin, MA). Amino acid (AA) contents of diets were determined using a High Performance Liquid Chromatography system (Agilent 1200 HPLC equipped with an Agilent1200 Series diode detector, Santa Clara, CA) following procedures of AOAC (AOAC, 2006; method 982.30 E, a,b,c). For all AA except cysteine, methionine and tryptophan, dietary samples were hydrolyzed in 6 mol/L HCl for 24 h at 110°C prior to injection. A β-amino-n-butyric acid and ethanol amine mixture was used as the internal standard. Fatty acids were analyzed using gas chromatography (Varian 3800 GC; Varian Analytical Instrument, Walnut Creek, CA) and flame ionization detector. The response factor for each peak was identified using

an internal standard composed of 28 fatty acids (462 standard, Nu-Check Prep, Inc., Elysian, MN).

Table 1.2. Ingredient composition and analyzed proximate nutrient profile of experimental diets.

Ingredients	Diet (g/100 g diet)			
	1	2	3	4
Wheat flour[1]	28.50	21.55	14.50	7.55
Fish meal[2]	21.00	22.25	23.50	24.75
Soybean meal - defatted[3]	16.60	16.60	16.60	16.60
Porphyra[4]	14.00	14.00	14.00	14.00
Krill meal[5]	11.00	11.00	11.00	11.00
Alginate[6]	5.00	5.00	5.00	5.00
Diatomaceous earth[7]	1.80	7.65	13.60	19.40
Vitamin mix[8]	1.00	1.00	1.00	1.00
Menhaden fish oil[9]	0.45	0.45	0.45	0.45
Corn oil[10]	0.45	0.30	0.15	0.05
Cholesterol[11]	0.20	0.20	0.20	0.20
Analyzed composition (% dry matter basis)				
Dry matter	48.8	48.9	47.1	45.3
Crude protein	40.4	40.9	39.8	39.6
Crude fat	6.1	5.7	5.8	6.1
Ash	11.2	17.1	23.0	28.2
Gross energy (kcal/g)	4.63	4.31	4.10	3.85
Protein to energy ratio (mg/kcal)	87.2	94.9	96.9	102.9

[1]Hawaiian Flour Mill, Honolulu, HI.

[2]RMI Fishmeal, Republic of the Marshall Islands

[3]Land-o-Lakes, Seattle, WA.

[4]Porphyra yezoensis (powder), Global Maxlink LLC, Antelope, CA.

[5]Florida Aqua Farms Inc., Dade City, FL.

[6]Sigma-Aldrich, Louis, MO.

[7]Hawaiian Hydroponics, Honolulu, HI.

[8]MP Biomedical LLC, Solon, OH. nicotinic acid (3.00 g/kg), D-calcium pantothenate (1.60 g/kg), pyridoxine HCl (0.70 g/kg), thiamine HCl (0.60 g/kg), riboflavin (0.60 g/kg), folic acid (0.20 g/kg), D-biotin (0.02 g/kg), vitamin B12 (0.1% triturated in mannitol) (2.50 g/kg), a-tocopherol powder (250 U/gm) (30.00 g/kg), vitamin A palmitate (250,000 U/gm) (1.60 g/kg), vitamin D3 (400,000 U/gm) (0.25 g/kg), phylloquinone (0.075 g/kg), and powdered Sucrose (959.655 g/kg)
[9]Virginia Prime Gold Menhaden Fishoil, Omega Protein Corporation, Houston, TX.

[10]Local supermarket, Honolulu, HI.

[11]Zeigler Brothers Inc., Gardners, PA.

Table 2.2. Amino acid composition of the experimental diets and pooled soft body tissue (SB) (% dry matter).

Amino Acid	Diet				SB
	1	2	3	4	
Alanine	7.40	7.84	7.83	7.84	5.70
Asparagine + Aspartate	10.10	10.05	10.32	10.06	15.34
Cystine	3.53	1.79	2.08	3.42	3.25
Glutamate + Glutamine	12.16	12.40	11.91	11.17	12.08
Glycine	6.62	6.95	6.86	7.55	8.72
Proline	5.45	5.36	5.14	5.16	4.18
Serine	4.37	4.52	4.49	4.38	3.96
Tyrosine	3.48	3.55	3.53	3.44	2.09
Taurine	1.05	1.06	1.07	1.04	3.74
Arginine	7.03	7.47	7.39	7.35	8.80
Histidine	2.71	2.73	2.85	2.74	1.30
Isoleucine	4.41	4.43	4.41	4.28	4.05
Leucine	7.26	7.32	7.33	7.10	7.22
Lysine	7.10	7.10	7.34	7.30	8.43
Methionine	3.07	2.99	3.14	3.17	3.29
Phenylalanine	4.69	4.41	4.41	4.34	3.94
Threonine	5.01	5.36	5.30	5.20	3.63
Valine	5.62	5.73	5.69	5.51	4.02

Table 2.3. Fatty acid composition of the experimental diets and pooled soft body tissue (SB) (% dry matter).

Fatty acid	Diet				SB
	1	2	3	4	
Octanoic acid (C8:0)	0.03	0.03	0.03	0.03	0.03
Decanoic acid (C10:0)	0.26	0.05	0.06	0.05	0.29
Dodecanoic acid (C12:0)	0.44	0.54	0.59	0.39	0.59
Tetradecanoic (Myristic) acid (C14:0)	4.72	4.92	5.79	4.34	3.75
Pentadecanoic acid (C15:0)	0.70	0.73	0.83	0.62	1.55
Palmitic acid (C16:0)	30.42	29.79	32.22	27.96	34.32
Palmitoleic acid (C16:1n-7)	3.60	3.80	4.40	3.43	0.84
Hexadecenoic (trans-Palmitilaidic) acid (C16:1n-9)	0.85	0.88	0.97	0.78	0.76
Heptadecanoic acid (C17:0)	0.27	0.30	0.35	0.28	0.05
Hexadecadienoic acid (C16:2n-4)	0.13	0.16	0.18	0.16	0.12
Hexadecatrienoic acid (C16:3n-4)	0.36	0.36	0.43	0.35	7.41
Stearic acid (C18:0)	4.31	4.29	4.46	3.97	5.65
Oleic acid (C18:1n-9)	10.08	9.64	9.85	10.04	6.05
Octadecenoic acid (C18:1n-7)	3.30	3.22	3.33	3.11	4.32
Linoleic acid (C18:2n-6)	10.04	9.53	8.08	11.93	0.89
Gamma Linolenic acid (C18:3n-6)	0.12	0.13	0.13	0.12	0.00
alpha-Linolenic acid (C18:3n-3)	1.68	1.70	1.67	1.77	0.76
Eicosanoic (C20:0)	2.13	2.08	2.20	2.01	0.35
Eicosenoic acid (C20:1n-9)	0.00	0.00	0.00	0.00	0.00
Eicosatrienoic acid (C20:3n-3)	0.84	0.95	0.75	1.09	0.00
Eicosatetraenoic acid (C20:4n-3)	0.21	0.22	0.18	0.22	0.99
Arachidonic acid (C20:4n-6)	0.60	0.62	0.58	0.59	5.85
Eicosapentaenoic acid (C20:5n-3)	13.52	13.68	12.21	13.74	10.40
Docosapentaenoic acid (C22:5n-3)	0.27	0.28	0.22	0.31	0.18
Docosapentaenoic acid (C22:5n-6)	0.14	0.17	0.12	0.21	0.59
Docosahexaenoic acid (C22:6n-3)	3.73	4.16	2.90	4.71	0.00

2.2.3. Growth performance trial

A 180-day growth performance trial was conducted in a semi-indoor facility, similar to a greenhouse with natural venting and airflow. Air temperature in the laboratory (24.6 to 29.3 °C) was continuously recorded in 6 second intervals using HOBO MX1101 Temperature/Relative Humidity Data Logger (Accuracy ±0.2 °C, Onset Computers, Bourne, MA). Ambient seawater temperature (25.6 to 27.4 °C) and dissolved oxygen (4.41 to 6.37 mg/L) was measured twice daily and averaged using an YSI Pro20 Dissolved Oxygen Meter (Accuracy ± 0.3 °C and ± 0.2 mg/L, YSI Inc., Yellow Springs, OH). The pH (7.89 to 8.25) was measured once daily using an EcoSense pH10A instrument (YSI Inc., Yellow Springs, OH), and salinity (31 g/L) was measured once daily using a Vital Sine Refractometer (Accuracy ±1.0%, Pentair, Apopka, Florida). The photoperiod was maintained at 13:11 h (Light:Dark) to simulate a non-spawning environment.

Out of 90 collected yellowfoot limpets, a total of 60 animals with mean initial weight 5.9 ± 1.72 g and length 33.9 ± 2.13 mm were selected and stocked in a completely randomized design. There were a total of 5 tanks (75 cm × 30 cm × 30 cm) assigned to each dietary treatment, 3 replicate (animals) nested in each tank, and a single animal per plate. Each animal was restricted to its own vertically suspended acrylic plate (15 cm length × 15 cm width × 2 sides = 450 cm^2 total surface area) with 50% surface area above and 50% surface area below the water level. Animals were allowed to crawl on either side of the plate. Overhead inlets were plumbed with irrigation nozzles providing spray (1 L/min flow rate) to air-exposed surfaces to prevent desiccation and osmotic stress.

Animals were fed once daily 1600 h by adhering a pre-weighed piece of feed on each plate above the water level to prevent leaching of nutrients. The flow rate was reduced overnight to

maintain feed moisture levels and reduce feed loss. Feed intake was assessed the following morning at 0800 h by subtracting the remaining unconsumed moist feed (g) from the offered moist feed ration (g). The daily feed ration started at 0.2 g and increased to 6.5 g; and was increased by 0.1 g when >50% of animals consumed 100% of ration from the previous night. This criterion prevented excessive waste of feed as feeding intervals reached up to 5 days, especially in the first half of the experiment.

Animals were measured prior to stocking, as well as every 30-day period for shell length (mm), shell width (mm), and total wet weight (g) using a dial caliper (0.1 mm, Empire Level) and electronic scale (0.0001 g, AG104 Mettler-Toledo International, Columbus, OH). Individuals were detached from each plate using a metal putty knife, placed on a towel to remove excess water, and measured. Mortalities were recorded and discounted for respective 30 days and 180 days calculations. At the end of the trial, surviving animals were sacrificed for soft body tissue measurements by removing the shell via dissecting knife.

2.2.4. Calculations

Recorded data and measurements were used to calculate average daily feed intake (ADFI), average daily gain (ADG), specific growth rate (SGR), shell length increment (SLI), shell width increment (SWI), feed conversion efficiency (FCE), protein efficiency ratio (PER), and soft body to shell ratio (SB/S). Calculations were done using wet masses; and protein intake was based on feed that was consumed by animals with respect to dietary proximate composition analyses.

ADFI = feed intake (wet g) / t (d)

$ADG = Wt_f - Wt_i / t$ (d)

FCE = SGR/ADFI

PER = Wt_f-Wt_i / protein intake (g)

SGR (%BW d^{-1}) = Ln (Wt_f) – Ln (Wt_i) / t (d) ×100

SLI (µm d^{-1}day) = SL_f (mm) – SL_i (mm) / t (d) × 1000

SWI (µm d^{-1}) = SW_f – SW_i / t (d) × 1000

SB/S = Soft body tissue (wet g) / Shell (g)

2.2.5. Statistical analyses

Statistical analyses were done using SAS (SAS v9.2, SAS Institute Inc., Cary, NC). Treatment effect was analyzed by one-way ANOVA using MIXED procedure of SAS. Differences among individual treatment means were tested by Tukey-Kramer test and means were separated using pdmix macro of SAS. The differences were considered to be significant at a probability level of 0.05.

2.3. Results

The growth performance measurements (ADFI, ADG, FCE, and PER) are reported in Table 2.4. Average daily feed intake ranged from 0.07 to 0.10 g at the start of the trial, and ranged from 4.31 to 4.54 g at the end of the trial. There were significant differences ($p < 0.05$) in ADFI in period d91-120 and d121-150. There were also linear effects of dietary treatment on ADG ($p <$

0.05), FCE ($p = 0.05$), and PER ($p < 0.05$) for d0-180, and quadratic effects of dietary treatment on FCE ($p < 0.05$) and PER ($p = 0.05$) for d0-180.

Growth rates (SGR, SLI, and SWI) are presented in Table 2.5. There was a linear effect ($p < 0.05$) of dietary treatment on SGR for d151-180; and a significant effect ($p < 0.05$) on SGR for overall study period (d0-180). The SGR of Diet 1 (0.12 %BW/d) was significantly higher than that of Diet 4 (0.01 %BW/d), but not Diet 2 (0.08 %BW/d) and Diet 3 (0.05 %BW/d). There was no significant difference in SWI ($p > 0.05$); however, there was both linear and quadratic effect ($p < 0.05$) of dietary treatments on SLI for d0-180. SLI decreased from 15.41 µm/d for Diet 1 to 6.60 µm/d for Diet 4.

The relative softbody tissue to shell mass ratio (g/g) ranged from 0.45 to 0.55. There were no significant differences ($p > 0.05$) between dietary treatments.

Survival for each 30-day period was good (75-100%) for all dietary treatments, with the exception of Diet 2 during Day0-30 (40%) (Figure 2.2.).

Table 2.4. The average daily feed intake (ADFI), average daily gain (ADG), feed conversion efficiency (FCE), and protein utilization of limpets fed experimental diets. The standard error of the mean (SEM) and *p*-values are reported.

Variable	Diet				SEM	P-value		
	1	2	3	4		Main	Linear	Quadratic
ADFI (g/d)								
Day0-30	0.07	0.10	0.07	0.07	0.01	0.28	0.73	0.70
Day31-60	0.13	0.13	0.12	0.12	0.00	0.96	0.73	0.82
Day61-90	0.31	0.30	0.28	0.32	0.01	0.46	0.94	0.39
Day91-120	1.35	1.34	1.04	0.90	0.11	0.00	<.0001	0.00
Day121-150	2.23	2.08	1.80	1.51	0.16	<.0001	<.0001	<.0001
Day151-180	4.54	4.31	4.49	4.37	0.05	0.10	0.26	0.36
Day0-180	1.21	0.70	1.04	0.82	0.11	0.15	0.24	0.37
ADG (mg/d)								
Day0-30	-2.14	2.22	-5.00	-6.92	1.99	0.24	0.11	0.13
Day31-60	3.57	-5.56	2.50	-1.54	2.08	0.26	0.62	0.65
Day61-90	1.43	-7.14	5.83	-4.55	2.93	0.36	0.83	0.46

Variable	Diet				SEM	P-value		
	1	2	3	4		Main	Linear	Quadratic
Day91-120	3.33	-2.00	-6.67	4.44	2.57	0.42	0.96	0.36
Day121-150	13.64	2.00	5.00	1.11	2.86	0.55	0.26	0.49
Day151-180	12.73	2.50	-5.00	-10.00	4.94	0.17	0.04	0.17
Day0-180	6.36	5.00	2.50	0.00	1.41	0.16	0.03	0.08
FCE								
Day0-30	114.32	184.94	111.55	84.60	21.44	0.64	0.54	0.49
Day31-60	103.47	108.24	106.58	52.63	13.40	0.66	0.32	0.22
Day61-90	74.40	34.27	69.28	58.11	8.92	0.70	0.90	0.97
Day91-120	16.35	14.67	11.84	10.85	1.27	0.82	0.36	0.54
Day121-150	12.77	16.58	13.34	11.70	1.05	0.89	0.68	0.54
Day151-180	4.94	6.20	4.79	4.14	0.43	0.94	0.63	0.62
Day0-180	8.43	9.22	7.64	3.63	1.24	0.18	0.05	0.04

Variable	Diet				SEM	P-value		
	1	2	3	4		Main	Linear	Quadratic
PER								
Day0-30	4.76	7.78	4.29	4.00	0.87	0.66	0.60	0.61
Day31-60	4.53	5.29	4.32	2.36	0.62	0.67	0.29	0.23
Day61-90	2.87	1.74	3.48	2.64	0.36	0.47	0.75	0.95
Day91-120	0.79	0.76	0.53	0.86	0.07	0.80	1.00	0.53
Day121-150	0.63	0.82	0.63	0.52	0.06	0.72	0.42	0.31
Day151-180	0.25	0.27	0.21	0.24	0.01	0.98	0.79	0.96
Day0-180	2.38	2.63	1.87	1.11	0.34	0.17	0.03	0.05

Table 2.4. The specific growth rates (SGR), shell length increment (SLI), and shell width increment (SWI) of yellowfoot limpet fed experimental diets. The standard error of the mean (SEM) and p-values are reported.

Variable	Diet				SEM	*P*-value		
	1	2	3	4		Main	Linear	Quadratic
SGR (% BW/d)								
Day0-30	-0.03	0.07	-0.06	-0.11	0.04	0.14	0.09	0.08
Day31-60	0.03	-0.16	0.03	-0.02	0.04	0.24	0.89	0.87
Day61-90	0.03	-0.19	0.09	-0.09	0.06	0.37	0.85	0.60
Day91-120	0.04	-0.01	-0.13	0.02	0.04	0.61	0.71	0.68
Day121-150	0.20	0.07	0.09	0.05	0.03	0.76	0.40	0.61
Day151-180	0.19	0.08	-0.06	-0.15	0.08	0.12	0.02	0.10
Day0-180	0.12	0.08	0.05	0.01	0.02	0.06	0.01	0.04
SLI (µm/day)								
Day0-30	7.14	7.41	8.33	10.26	0.71	0.96	0.62	0.63
Day31-60	2.38	3.70	0.00	0.00	0.92	0.50	0.20	0.36
Day61-90	21.43	11.90	19.44	13.64	2.28	0.63	0.53	0.56
Day91-120	23.61	10.00	9.72	7.41	3.69	0.27	0.11	0.39
Day121-150	21.21	26.67	20.00	3.70	4.95	0.31	0.12	0.06
Day151-180	9.09	16.67	12.50	2.08	3.08	0.37	0.27	0.10
Day0-180	15.41	14.58	13.54	6.60	2.01	0.15	0.04	0.03

Variable	Diet				SEM	*P*-value		
	1	2	3	4		Main	Linear	Quadratic
SWI (μm/day)								
Day0-30	21.43	18.52	25.00	25.64	1.66	0.85	0.47	0.57
Day31-60	7.14	0.00	0.00	2.56	1.68	0.18	0.23	0.95
Day61-90	21.43	16.67	22.22	21.21	1.26	0.96	0.87	0.89
Day91-120	12.50	20.00	2.78	11.11	3.53	0.21	0.35	0.92
Day121-150	18.52	26.67	18.33	5.56	4.36	0.30	0.14	0.07
Day151-180	9.09	8.33	4.17	0.00	2.10	0.39	0.09	0.15
Day0-180	14.90	18.06	14.24	10.76	1.50	0.47	0.18	0.14

Figure 2.1. Weekly air temperature during 180-day study period (mean ± standard deviation).

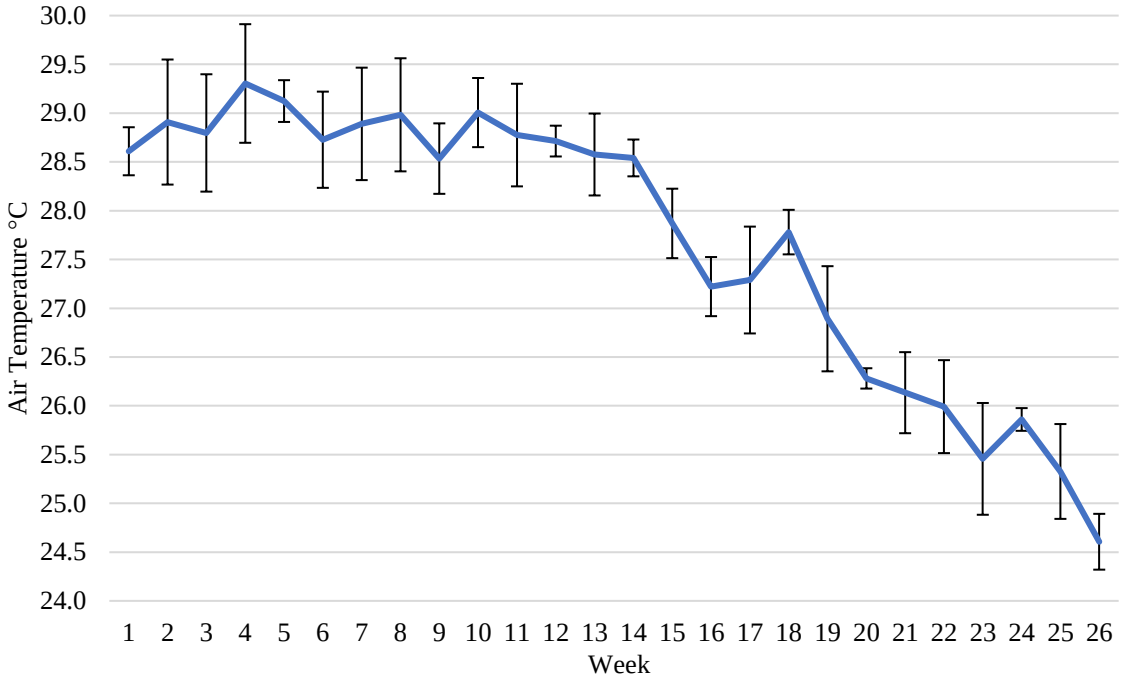
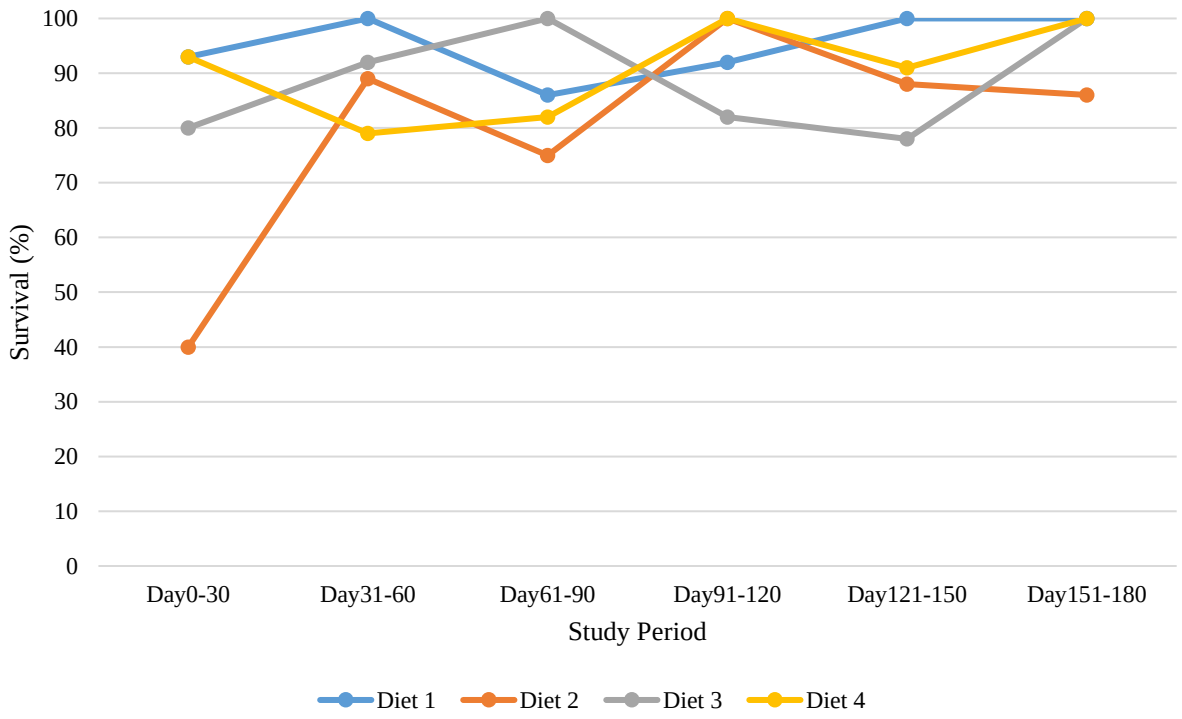


Figure 2.2. Percent survival of yellowfoot limpet during 180-day study period.



2.4. Discussion

The overall design of this grow-out system allowed for successful rearing of yellowfoot limpet with improved survival rates, which was a major issue during past trials of Hua and Ako (2016). In terms of feeding strategy, this design improved the capacity for culturing limpets that graze above sea level in the intertidal.

In the feeding trial, there was notable change in feed behavior with respect to ADFI. Prior to period d91-120, limpets were exhibiting intermittent, non-feeding days of up to four days. However, this pattern shifted to daily feeding, as well as a 3-4-fold increase in ADFI for all treatments from d61-90 to d91-120. It was considered feed acclimation to be a possible explanation, however, these animals were accustomed to the feed weeks prior to the start of the trial. Instead, this change in feeding behavior was observed to coincided with a decrease in air temperature from 28.5 °C to 27.9 °C in week 15 (Figure 2.1.). This inverse relationship between temperature and ADFI remained for the rest of the study period. Although air temperature was not considered as a main factor, it is reasonable to say ambient air temperatures were not in the optimal range for limpets during the first 3 periods (d0-30, d31-60, and d61-90). Similar to this study, optimal temperatures for abalone (*H. midae)* were found to parallel the range of mean seawater temperature of their natural habitat; and when the water temperature was elevated outside of that range, the feed consumption, growth, and feed utilization decreased (Britz *et al.* 1997). Additionally, low survival for Diet 2 during the first period suggested hot summer-like temperatures may have negatively impacted performance. Although metabolism was not monitored, it seems that animals showed signs of shifting metabolism as ambient air temperature changed. Mortalities showed what appeared to be oxygen depletion (pale or discolored foot muscles), poor osmoregulation (shrunken mantle tissues), and possible infections (foot atrophy);

all similar physiological responses to temperature that were reviewed for abalone (Morash and Alter, 2015). A study by Parry (1978) also found that metabolism of a cousin limpet, *Cellana tramoserica*, were caused by temperature mediated growth rates, and that these particular snails do not acclimate to seasonal changes in temperature. For yellowfoot limpet, metabolism may have increased uncontrollably during warmer daytime hours, ultimately causing the breakdown of their energy reserves or foot muscle, decreasing feed utilization, and stunting growth early on in the trial. As temperature decreased in the system, animal metabolism shifted favorably, and growth performance improved.

As far as the effect of energy level on growth performance, there were no significant differences between treatments for ADG, FCE, and PER. In a related PE ratio study, Bautista-Teruel and Millamena (1999) reported best FCE (1.50 ± 0.08) and PER (2.47 ± 0.05) for the highest PE dietary treatment (31.48% CP/3090 kcal/kg ME; 101.88 mg/kcal) for abalone (*H. asinine*); and authors considered that low energy dietary treatment animals had higher feed intake to compensate for dietary energy deficiencies. In this study, all treatments were offered constant dietary protein level (40%), but animals offered lower energy diets did not overcompensate by increasing feed intake.

These results indicate that basal PE ratio demand was met for all diets; however, higher energy diets performed better. This means that protein was spared by increasing carbohydrate energy sources. The SGR across dietary treatments ranged from 0.01 to 0.12 %BW/d over 180 days with peak SGR of 0.20 %BW/d, which followed a significant increase in ADFI in the fourth period of trial (d91-120).

As far as growth rates, animals on Diet 1 had a significantly higher SGR (0.12 %BW/d) compared to Diet 4 (0.01 %BW/d) (see Table 2.5.). In the last two months of trial, animals on

Diet 1 had 0.20 %BW/d, which compares to that of abalone (Uki, 1985; Nelson *et al.* 2002). SGR for all treatments increased dramatically from d91-120 to d121-150, which followed the increase in ADFI. In fact, animals on all treatments appeared to sustain minimal or negative growth during the first four periods of trial when feeding was marginal. The poor growth pattern observed are nearly identical to that in the wild, where yellowfoot limpet exhibited decreased growth rates from May through October (Kay *et al.* 2006). For all treatments, the SGR increased over a short period of time, which indicates that growth rates for yellowfoot limpet are dynamic. Shell length increment for the entire study period decreased linearly from Diet 1 (15.41 µm/d) to Diet 4 (6.60 µm/d), and followed the other growth measurement trends.

The slight decrease in SGR, SLI, and SWI for the last month are indicative of a possible shift in growth from somatic to reproductive – though these are not mutually exclusive. Although we did not investigate maturation, it can be noted that natural spawning season of limpet (October to January) coincided with last half of this study period. Also, 51.7% of the study population had gonads at the end of the study. Kay (1977) reported the earliest onset of maturation for yellowfoot limpet to be 20-25 mm SL; and in terms of optimizing somatic growth rates for limpet production. Based on this information, it can be suggested to use juvenile animals <20 mm to ensure that there is no influence of reproductive growth. However, due to local fishing laws, wild limpet collected below 31 mm SL would require permitting. Henceforth, the production of captive reared juveniles for this type of research would benefit our understanding of limpet nutritional demands.

Moreover, according to Kay *et al.* (2006), yellowfoot limpet gonad constitutes up to 46.5% of soft body weight in spawning season. The relative soft body to shell mass ratio found in this study indicates that nearly half of the total limpet weight come from shell mass. Thus, it can be

suggested that the market value of limpet sold by weight may be drastically impacted by these factors (i.e. gonad development and relative soft body to shell mass). And local preference is for limpets with gonads due to the rich flavor of these fat-rich tissues, which supports understanding both somatic and reproductive growth for this species.

2.5. Conclusions

Overall, yellowfoot limpets performed indifferently with respect to diets with constant 40% protein level and staggered energy levels. Although there were no significant effects of diet on growth performance across the 180-day period, the highest energy diet (Diet 1, 87.2 mg/kcal) maintained the best overall growth. A diet with increased non-protein sources of energy may improve growth performance by freeing protein sources of energy for muscle growth. Growth rates also appear to change seasonally – dependent on the temperature regime. Temperature should be controlled/maintained in the optimal thermal range in future growth performance studies.

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Chapter 3

The reported occurrence of hermaphroditism in the yellowfoot limpet (*Cellana sandwicensis Pease*, 1981)

Abstract

The yellowfoot limpet *Cellana sandwicensis*, ‘opihi ‘alinalina, is a significant cultural resource and seafood for Hawaiians. With wild stocks greatly depleted and under significant commercial pressures, research efforts are focused on understanding their reproductive biology to improve management and ensure survival of this species.

During spawning season, wild aquaculture-housed limpets were examined on a monthly basis for sexual maturation, via gonadosomatic index and histological examination. Spawn events, which coincided with peak maturation, were observed. Furthermore, a single specimen was observed to be a hermaphrodite. While hermaphroditism has been a documented reproduction strategy for other *Patellid spp*., it has never been documented in any of the Hawaiian limpets (*Cellana spp*.).

Previously, *Cellana sandwicensis* was assumed to be gonochoristic with males and females. And despite sex ratios shifting across time and geographic location, no clear biological explanation existed for this phenomenon.

From this finding, we propose that the yellowfoot limpet may be a sequential hermaphrodite, which has, until now, gone undetected. This hermaphrodite, although carrying both gonads simultaneously, was most likely caught at an inter-sex level. It is then plausible to consider environmental factors (i.e. presence of conspecifics) playing a role in sex determination for this species.

In the future, defining sex ratios at various class sizes, time points, and geographic locations will improve our understanding of reproductive biology for yellow foot limpets. Moreover, monitoring individuals across multiple spawning events/seasons will clarify any speculation regarding their ability/inability to switch sexes.

3.1. Introduction

Nearly all forms of sexual reproduction have been documented for mollusks. In terms of hermaphroditism, some mollusks exhibit simultaneous hermaphroditism, sequential hermaphroditism, or alternating hermaphroditism reproductive strategies (Collins, 2013). In other cases, the ovo-testis, a small hermaphroditic duct containing both male and female gonad characteristics, is common (Lee *et al.* 2015). Despite this plastic reproductive molluscan nature, only 3% of known species of Eogastropods (formerly Prostobranchs) (Ponder & Lindberg, 1997) are functional hermaphrodites (Heller, 1993). Moreover, hermaphroditism occurs in less than 0.29% of all Patellid species, which is considerably rare (Dodd, 1956). For *Patella ferruginea*, a well-documented limpet, the minimum size at which sex change occurs is 40-60 mm shell length, and in the male to female direction. For protandrous species like this, size selecting (during harvesting) may negatively impact reproductive output and threaten species survival (Espinosa *et al.* 2006). Additionally, selecting larger individuals has been reported to cause mediated sex change in smaller sized individuals, which shifts population dynamics (Rivera-Ingraham *et al.* 2011). These aspects of hermaphroditism are considerably important for conservation and management efforts for at-risk Patellid species.

The yellowfoot limpet, *Cellana sandwicensis*, ‘opihi ‘alinalina an at-risk Patellid species, may also exhibit a form of hermaphroditism. Previous research reports that mature limpets maintain separate sexes, gonochoristic. The typical spawning season coincides with the peak of winter, however, occasional spawning events have been recorded during other times of the year (Kay *et al.* 1982). The peak of this spawning season is thought to shift year-to-year depending on the hydrodynamic environment (i.e. temperature, tide, and waves). The sex ratio shifts from being dominated by an unidentifiable sex during resting periods to a near 1:1 male to female ratio in spawning periods (Kay *et al.* 1982; Hua & Ako, 2014). However, this interesting phenomenon still lacks a biological explanation. Henceforth, authors aim to show evidence of a bi-directional form of sequential hermaphroditism, and to explain how hermaphroditism may be a significant reproduction strategy for the survival of this Hawaiian limpet.

3.2. Materials and methods

Yellowfoot limpets (*Cellana sandwicensis*) were collected from Rabbit Island, Oahu, Hawai’i (21°19′52″N 157°39′32″W) to monitor sexual reproduction. Animals (n=45) were stocked at random into a circular flow-through aquaculture system (total tank surface area of 0.6 m^2) and reared at ambient conditions.

Air temperature (18.51-33.57 °C) was recorded in 4 second intervals using HOBO MX1101 Temperature/Relative Humidity Data Logger (Accuracy ±0.2 °C, Onset Computers, Bourne, MA). Seawater temperature (24.65-26.93 °C) and dissolved oxygen was measured twice daily and averaged using an YSI Pro20 Dissolved Oxygen Meter (Accuracy ± 0.3 °C and ± 0.2 mg L^{-1}, YSI Inc., Yellow Springs, OH). Seawater pH (8.24) was measured once daily using an

EcoSense pH10A instrument (YSI Inc., Yellow Springs, OH), and salinity (30 mg/L) was measured once daily using a Vital Sine Refractometer (Accuracy ±1.0%, Pentair, Apopka, FL).

Animals were offered a formulated diet (*ad libitum*) with access to feed between 16:00 and 08:00 hours. Animals (n=3) were sacrificed monthly to determine maturation status, and at the end of the trial. The reported hermaphrodite specimen was sacrificed at the end of the trial (May), and dissection of this individual revealed the presence of both ovary and testis.

Measurements for shell length and shell width (mm) were done using an electronic caliper (0.01 mm), and measurements for total weight (g), soft-body weight (g), and gonad weight (g) used an electronic scale (0.0001 g, AG104 Mettler-Toledo International, Columbus, OH). The gonadosomatic index (including both ovary and testis) was calculated as follows: GSI = [total gonad weight / total soft body weight] x 100.

Gonad tissue was fixed in 10% v/v neutral buffered formalin for 24 hours at room temperature and rinsed with 70% v/v ethanol. Samples were wax embedded, and 6 µm horizontal sections across the entire length of the gonad were stained with haematoxylin and eosin. Images were captured with Infinity Analyze V 6.5.4 Lumenera Software and Infinity3S-1UR microscopy camera (Lumenera Corporation, Ottawa, ON), and compound microscope Olympus BX43 (Olympus, Center Valley, PA).

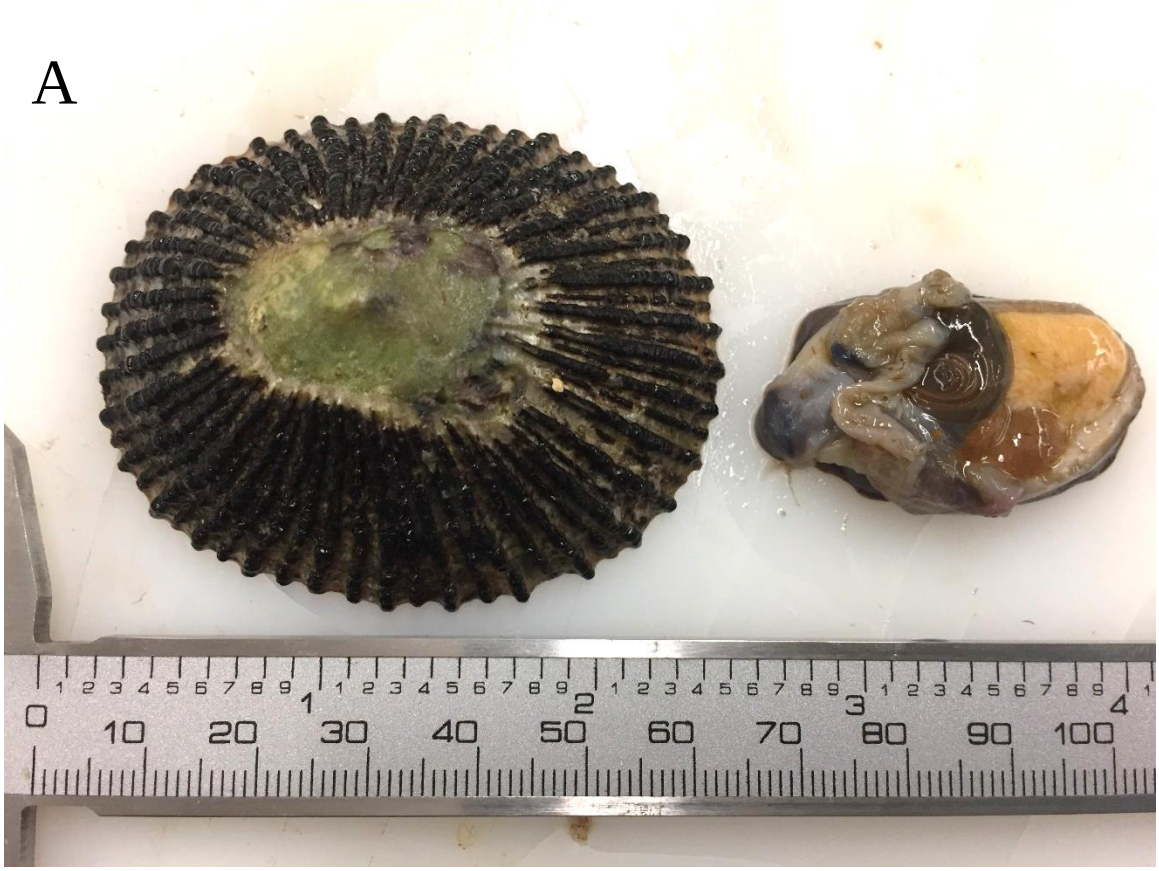
3.3. Results and Discussion

The sex ratio during the current spawning season was 1:3 male to female. The sex ratio during this study differs from previous literature, which has previously been reported to be 1:1 male to female in two separate studies (Kay *et al.* 1982; Hua & Ako, 2014). This may be due to different determinants of sex: environment (i.e. conspecific interaction, temperature and pollution/pollutants) and/or genetics found across different time scales and geographic locations.

Recently, we surveyed yellowfoot limpets in the Papahanaumokuakea Marine National Monument (a marine reserve in the Northwestern Hawaiian Islands), which appeared to be another egg-rich population (unpublished data); and we postulate that the presence of larger individuals and/or high-density aggregates may be factoring in to this phenomenon. More specifically, the sperm to egg ratio may play a role in controlling proper fertilization (Levitan, 2006), and therefore influence sex ratios.

During this study, only 1 of 45 samples was found to be hermaphroditic. The specimen's shell (Figure 3.1.) was 62.08 mm long and 49.54 mm wide, it is considered a relatively large individual (the average shell length and shell width for this collected group was 37.96 ± 3.76 mm and 29.91 ± 3.24 mm, respectively). The specimen's total weight was 35.54 g and soft body tissue weight was 10.75 g. The total gonad weight, including both ovary and testis, was 0.68 g; and the GSI was 6.33% at the time of dissection. In March 2017, this individual was observed spawning in the tank environment, naturally. We determined this specimen to be a functioning male based on the release of white colored gametes. However, the presence of female reproductive organs found during dissection provided evidence that this specimen should be classified as a hermaphrodite.

Figure 3.1. (A) Dorsal side of dissected hermaphrodite specimen. The ovary is a brown tissue mass located ventral to the visceral mass. The testis is the milky, white tissue mass located ventral to the visceral mass. (B) The ventral side of the hermaphrodite sample and muscular foot.



B

The small sample size and low rate of hermaphroditism (2.22%) may simply be a result of random, genetic mutation acting on this single specimen. However, we must also consider hermaphroditism as a viable reproduction strategy for this species. When considering the classification of hermaphrodites, yellowfoot limpets might be recognized as simultaneous hermaphrodites since both ovary and testis were observed in this specimen. However, histology indicates that this specimen was not a functioning simultaneous hermaphroditism. The testis of this specimen contained mature spermatozoa with gaps in the gonadal cavity indicating a mature/post-spawn stage of gonad development (Figure 3.3.). The ovary contained primordial germ cell proliferation, indicating an early stage of gonad development for future spawning events (Figure 3.2.). Because this individual had an immature ovary and functioned as a male during

spawning season, it would not be physiologically capable of selfing (self-fertilization) or cross-fertilizing with a mate – a recognizable characteristic of simultaneous hermaphrodites (Heller, 1993).

Figure 3.2. (A) Histological examination of the ovary tissue at 40x magnification. The ovary is in the pre-vitellogenic stage showing the surrounding hemocoel (HE), epithelial cells (EP) lining the digestive gland (not shown), the ovarian lumen (OL), and the traversing trabecula (TR) from which asymmetrical primordial germ cells (PGC) are connected. (B) Histological examination of the ovary tissue at 400x magnification. Primordial germ cells (PGC) are found connected to epithelial tissue of the trabecula (TR), which will enlarge and develop within the lumen of the trabecula until released as mature oocytes in the ovarian lumen (OL).

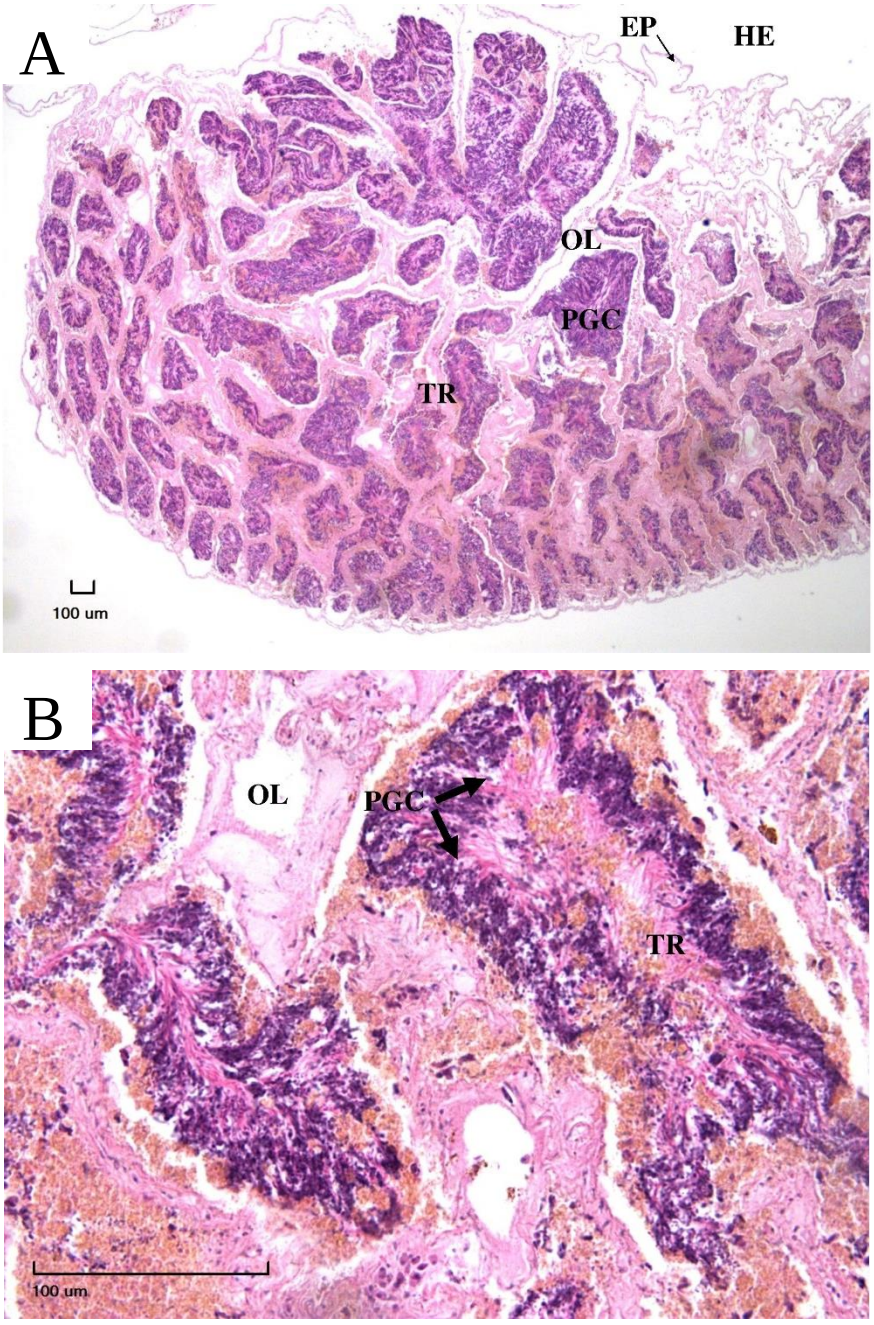
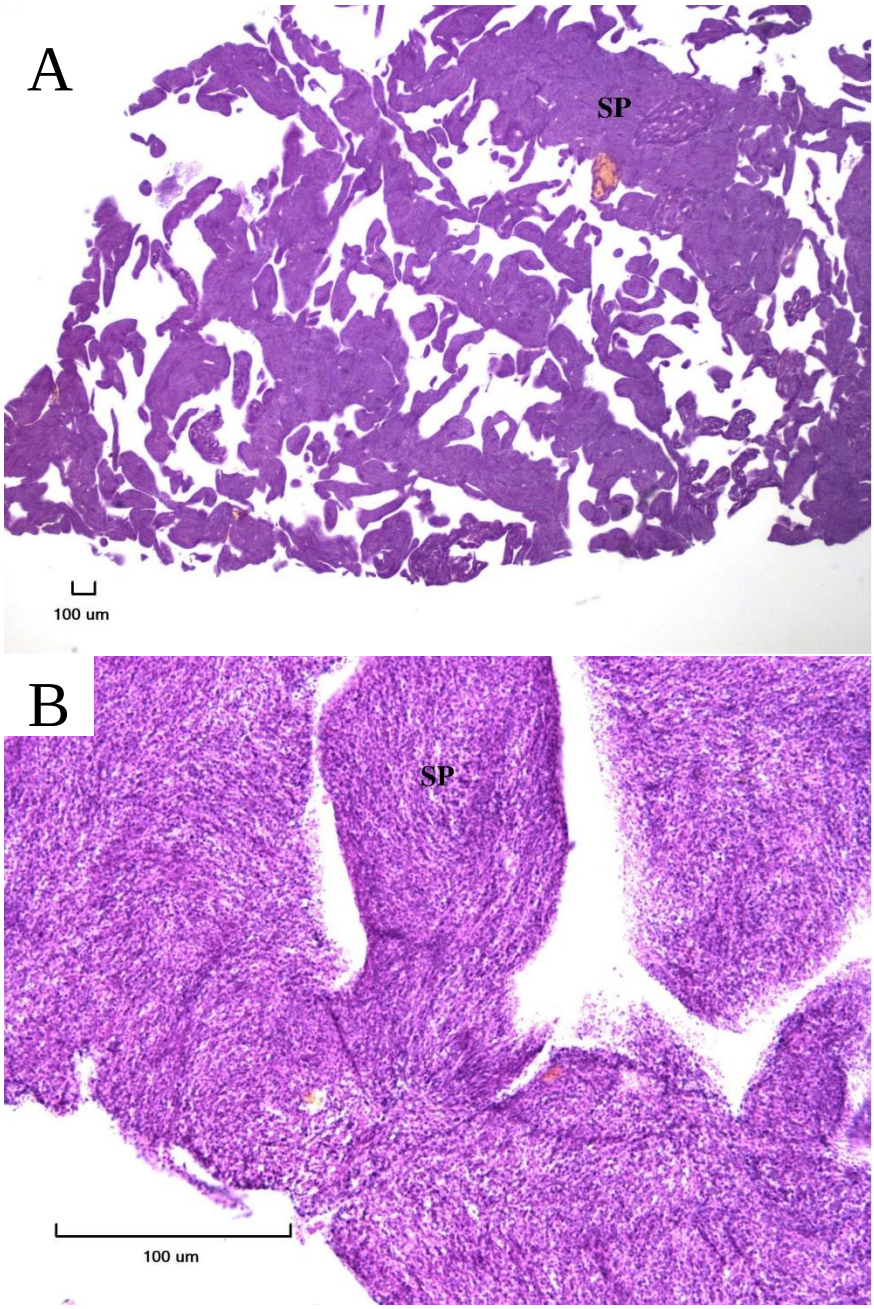


Figure 3.3. (A) Histological examination of the testis tissue at 40x magnification. Mature spermatozoa (SP) are found within the larger partially spawned testis. (B) Histological examination of the testis tissue at 400x magnification. Mature spermatozoa are found with dark, symmetrical nuclei.



The apparent sex switch occurring in this individual specimen is most likely a form of sequential hermaphroditism. However, based on current data for yellowfoot limpets, there is no evident bias in sex ratio at any given size class that would indicate this to be a protandrous or protogynous species (unidirectional sex change) (Allsop & West, 2004). A more feasible theory is that this species maintains a bi-directional form of sequential hermaphroditism – a reproductive strategy found in fish (Munday *et al.* 1998), oysters (Yasuoka &Yusa, 2017), and limpets (Guallart *et al.* 2013) – that allows switching of sexes in either direction based on environmental influences.

During sex change of sequential hermaphrodites, it is theorized that gonad development is arrested and the animal is not reproductively viable (Dodd, 1956). We explain that the discovery of a specimen carrying both male and female gonads may illustrate a rarely observed intermediate stage during the transition between sexes – where an individual would usually transition into an unidentifiable sex (reduced gonad cavity). We also consider that this specimen appears to sex-switch in a single spawning season, and may have continued developing into a functioning female without a resting phase between the transitions. This would mean that yellowfoot limpets can potentially sex-switch at any given time of the year. However, it appears that the "normal" period where we could expect the sex switching to occur would be between spawning seasons (resting period/offseason).

Interestingly enough, hermaphrodites that switch sex back and forth are only found in broadcast spawning species of marine snails (Collin, 2013). From an evolutionary perspective, the ability to switch sexes is crucial for the survival of the species when lacking copulatory organs. For yellowfoot limpet, an at-risk species, individuals capable of switching sex based on their available partners allows them to reproduce under adverse conditions (i.e. low densities). We therefore suggest these limpets most likely undergo environmental sex determination – where

individuals can influence the sexual status of another conspecific via pheromones – as opposed to genetic sex determination associated with other sequential hermaphrodites (Heller, 1993).

To test this speculative theory, researchers would need to monitor broodstock across multiple spawning seasons to determine if functional sex-switching occurs. And based on the low rate of hermaphroditism, a relatively large number of individuals would be required. Based on our postulated theory, altering the size class and/or densities in the tank environment over time may increase the rate of hermaphroditism. Additionally, frequently sampling throughout the year would potentially increase detectability of direct sex-change (intermediate stage) within a single spawning season.

3.4. Conclusion

The yellowfoot limpets (*Cellana sandwicensis*), is an important traditional cultural and food resource, and a candidate aquaculture species. As part of proper management, conservation, and production, it is imperative to understand the life cycle and mode of reproduction for these marine snails. In this current study, we report a paradigm-shifting discovery, a hermaphrodite individual carrying an ovary and testis. Previous to this study, it has been reported that individuals are of separate sexes, male or female, but hermaphroditism was never observed. Although there were reports of annual shifts in sex ratios, this phenomenon could not be explained. We propose a theory that the yellowfoot limpet is a sequential hermaphrodite and is capable of switching sex in a bi-directional manner dependent on environmental cues.

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Chapter 4

Maturation, spawning, and larval development of yellowfoot limpet (*Cellana sandwicensis* Pease, 1861) in aquaculture

Abstract

The yellowfoot limpet (*Cellana sandwicensis*) is a proposed candidate aquaculture species because of its depleted wild fishery and high socio-economic value. In this study, aquaculture methods were examined for broodstock maturation, synchronous spawning, and larval rearing to settlement in the laboratory. Wild limpets ripened in the laboratory were synchronously spawned in three separate trials (10-13% spawning success) by administering a two-step intramuscular injection of salmon gonadotropin releasing hormone analogue (sGnRHa). Fecundity was found to be linearly correlated to weight ($P = 0.043$), and the most fecund female spawner (24,135 eggs per gram body weight) resulted in 85% fertilization success, 85% hatching success, and 35.4% survival to settlement competency in 22.26 ± 1.32 °C and 30 mg/L salinity filtered seawater. Larval rearing was completed in stagnant, clear-water vessels; and rapid development from egg to metamorphic competency occurred over a span of 3.5 days. Late veliger larvae were observed to passively settle, and settlement occurred in the presence of biofilm. To the best of our knowledge, we present the first descriptions of yellowfoot limpet spawning behaviour, as well as larval development (illustrated) across 16 major stages from mature oocyte to postlarva.

4.1. Introduction

The world aquaculture production of molluscs accounted for 16.1 million tonnes of biomass and US$19 billion in sales, the second largest group to finfish in 2014 (FAO, 2016). According to a report from FAO (2016), a steady increase in mollusc production helped to offset a decline in natural abundances of key groups (i.e. abalone, clam, mussel, oyster), as well as to generate revenue for many countries. Molluscan aquaculture production will likely maintain an upward trend with both improvements to existing industries and establishment of new ones.

In terms of new aquaculture industries, Hawaiian limpets (*Cellana spp.*) are a highly prospective group due to their economic and cultural value (Mau and Jha 2017). Like many seafood industries, wild Hawaiian limpet production plateaued since human-harvesting collapsed the fishery (Kay and Magruder 1977; McCoy 2008). In particular, the yellowfoot limpet *Cellana sandwicensis* accounts for the majority of those catches, and is the preferred species valued at US$30-40 kg^{-1}. The yellowfoot limpet inhabits the lower intertidal zone – defined by crustose coralline algae – and is greatly exposed to tidal fluctuations and waves. They are widely distributed across the majority of islets/islands spanning from Northwestern Hawaiian Islands to main Hawaiian Islands, however, limited larval dispersal remains a concern for the conservation of this species (Bird *et al.* 2007).

In the early 1980's, the State of Hawai'i supported a project to culture Hawaiian limpets, which primarily focused on the blackfoot limpet *Cellana exarata*. From this project, Corpuz (1981) showed that the reproduction of *C. exarata* was possible by artificially removing their gametes and maturing the eggs for fertilization (strip spawning). Although other stress-induced-spawning techniques were examined, the author reported to have marginal success in controlling synchronous spawning of both sexes – a requirement for this broadcast spawning mollusc and

resorted to artificial maturation of oocytes. Although Corpuz (1981) successfully reared from juveniles to sexual maturity (ca. 6 months or 18 mm shell length), these spawning methods were impractical for aquaculture.

Recently, researchers applied hormone control to reproduce Hawaiian limpets. In one study, *C. sandwicensis* administered a series of weekly intramuscular injections of salmon-gonadotropin-releasing-hormone analogue (sGnRHa) reached final maturation after four weeks (Hua and Ako 2013). And, in a separate trial, authors reported to control synchronous spawning of gravid, wild limpets using a single intramuscular injection of sGnRHa (1000 ng g^{-1} BW). However, many aspects of reproduction and early life-history for *C. sandwicensis* remain unknown.

In terms of reproductive phenology, *C. sandwicensis* are understood to reach sexual maturity as early as 23-25mm shell length (Kay et al 1982). They have two potential spawning seasons with intermittent, non-spawning or resting periods each year. Based on gonadosomatic index (GSI) and recruitment of wild limpets, we understand there to be a well-defined, primary spawning season from October to April (Kay *et al.* 1982; Hua and Ako 2014). However, we have also observed wild limpets from various geographical locations to sustain large, ripe gonads from June to August, which indicates a potential secondary spawning season for some populations (unpublished). Despite there being two potential periods to spawn limpets (varying in time and space), we consider the strongest signal for reproduction of *C. sandwicensis* to be Winter for most populations.

The current study aimed to mature limpets in captivity (without hormone), control spawning, rear larvae, and describe early development of *C. sandwicensis* through metamorphosis. The present study will advance our understanding of the early life-history of this

species and maintains implications for both aquaculture development and management of this Hawaiian limpet.

4.2. Methods

4.2.1. Animal collection

The permitted collection of *C. sandwicensis* occurred on Manana Island (Oahu, HI, USA, 44.7064° N, 66.8158° W) during a predicted resting period (September). Adult limpets were selected for size (ca. 4 cm shell length) and carefully removed from rocky substratum using metal knifes. Limpets were transported – attached to black acrylic plates –within a few hours to the research facility. Upon arrival, animals were tagged, measured using a dial caliper (0.1 mm, Empire Level), and transferred to broodstock tanks.

4.2.2. Broodstock maturation

Adult limpets (n=135) were reared in an outdoor greenhouse in 95 L tanks (total surface area of 0.6 m^2) for six months. Each tank maintained a constant water-level at one-third of the tank height, which allowed limpets to inhabit areas above and below the air-water interface. A ring of artificial turf was lined at the lip of the tank to prevent limpets from escaping. Tanks were supplied ambient seawater in a flow-through manner by overhead, irrigation (360° spray) at 15 L/min.

Air temperature (18.51°C Min to 33.57°C Max) was continuously recorded in 6 second intervals using HOBO MX1101 Temperature/Relative Humidity Data Logger (Accuracy ±0.2°C, Onset Computers, Bourne, MA). Ambient seawater temperature (24.65°C Min, 26.93°C Max) and dissolved oxygen (5.15 mg/L Min to 6.87 mg/L Max) was measured using an YSI Pro20 Dissolved Oxygen Meter (Accuracy ± 0.3°C and ± 0.2 mg/L, YSI Inc., Yellow Springs, OH). Salinity (30 g/L) was measured once daily using a Vital Sine Refractometer (Accuracy ± 1.0%, Pentair, Apopka, Florida).

To feed animals, a maturation diet (Table 4.1.) was formulated and analyzed for composition following Mau and Jha (2018). All animals were offered feed between hours 16:00 and 08:00 *ad libitum*, and excess feed was removed the following morning.

Table 4.1.

Ingredient composition and analyzed nutrient profile of the formulated diet (% dry matter).

Ingredients	**Diet**
Wheat Flour[1]	29.00
Fish Meal[2]	21.00
Soy Meal – defatted[3]	16.60
Porphyra[4]	14.00
Krill Meal[5]	10.50
Alginate[6]	5.00
Diatomaceous Earth[7]	1.30
Vitamin mix[8]	1.00
Mineral mix[9]	1.00
Corn Oil[10]	0.25
Fish Oil[11]	0.15
Cholesterol[12]	0.20
Analyzed composition	
Dry matter	50
Crude protein	36
Crude fat	5
Ash	12
Gross energy (kcal/g)	4.56

[1]Hawaiian Flour Mill, Honolulu, HI.
[2]RMI Fishmeal, Republic of the Marshall Islands
[3]Land-o-Lakes, Seattle, WA.
[4]*Porphyra yezoensis* (powder), Global Maxlink LLC, Antelope, CA.
[5]Florida Aqua Farms Inc., Dade City, FL.
[6]Sigma-Aldrich, Louis, MO.
[7]Hawaiian Hydroponics, Honolulu, HI.
[8]MP Biomedical LLC, Solon, OH. nicotinic acid (3.00 g/kg), D-calcium pantothenate (1.60 g/kg), pyridoxine HCl (0.70 g/kg), thiamine HCl (0.60 g/kg), riboflavin (0.60 g/kg), folic acid (0.20 g/kg), D-biotin (0.02 g/kg), vitamin B12 (0.1% triturated in mannitol) (2.50 g/kg), a-tocopherol powder (250 U/gm) (30.00 g/kg), vitamin A palmitate (250,000 U/gm) (1.60 g/kg), vitamin D3 (400,000 U/gm) (0.25 g/kg), phylloquinone (0.075 g/kg), and powdered Sucrose (959.655 g/kg)
[9]MP Biomedical LLC, Solon, OH. calcium carbonate (35.7%), monopotassium phosphate (19.6%), potassium citrate monohydrate (7.078%), sodium chloride (7.4%), potassium sulfate (4.66%), magnesium oxide (2.4%), ferric citrate (0.606%), zinc carbonate (0.165%), manganese carbonate (0.063%), copper carbonate (0.03%), potassium Iodate (0.001%), sodium selenate, anhydrous (0.00103%), ammonium molybdate.$4H_2O$ (0.000795%), sodium metasilicate.$9H_2O$ (0.145%), chromium potassium sulfate.$12H_2O$ (0.0275%), lithium chloride (0.00174%), boric acid (0.008145%), sodium fluoride (0.00635%), nickel carbonate (0.00318%), and ammonium vanadate (0.00066%)
[10]Local supermarket, Honolulu, HI.
[11]Virginia Prime Gold Menhaden Fish oil, Omega Protein Corporation, Houston, TX.
[12]Zeigler Brothers Inc., Gardners, PA.

At the beginning of the study, animals that did not acclimate to our laboratory environment and/or were injured from collection were sacrificed to determine baseline maturation status. To determine maturation status, animals were sacrificed for gonadosomatic index (GSI) measurements; and GSI was calculated using following formula:

$$GSI = [\text{total gonad weight} / \text{total soft body weight}) \times 100$$

During the study, a minimum of n=6 animals were sacrificed in the beginning of each month to determine maturation status until remaining animals were used for spawning trials. The specific months, October to February, were selected based on our understanding of their natural reproduction cycle. For this species, external sexing is not possible, therefore, authors assumed the experimental group to be in a 1:1 female to male ratio.

Prior to spawning, gonad tissue (n=3) was fixed in 10% v/v Neutral Buffered Formalin for 24 hours at room temperature and rinsed with 70% v/v ethanol. Samples were wax embedded, and 6 µm horizontal sections across the entire length of the gonad were stained with haematoxylin and eosin. Images were captured with Infinity Analyze V 6.5.4 Lumenera Software and Infinity3S-1UR microscopy camera (Lumenera Corporation, Ottawa, ON), and compound microscope Olympus BX43 (Olympus, Center Valley, PA).

4.2.2.1. Statistical Analysis

Statistical analysis was done using SAS (SAS v9.2, SAS Institute Inc., Cary, NC). Monthly effect was analyzed by one-way ANOVA using MIXED procedure of SAS. Differences among individual treatment means were tested by Tukey-Kramer test and means were separated using

pdmix macro of SAS. The differences were considered to be significant at a probability level of 0.05.

4.2.3. Spawning

Three trials were carried out from February 20th to March 11th of 2017 (Table 2). For each spawn trial, a subset (Trial 1, n=16; Trial 2, n=37; Trial 3, n=70) of captive matured animals was selected at random. To control final maturation and synchronous spawning, sGnRHa (OVA-RH, Western Chemicals Inc., Syndel USA, Ferndale, WA, USA) was injected into the muscular foot of each limpet. The priming sGnRHa injection of 250 ng g^{-1} Body Weight (BW) was administered at least 24 hours prior to the resolving dose of 500 ng/g BW sGnRHa. As a control for each trial, we injected an additional subset (n=3) animals with phosphate buffered saline (PBS) with equivalent volumes to hormone injections on a BW-basis. For each trial, resolving injections were done at 20:00 h, and animals were immediately stocked into 1 L beakers or 19 L buckets depending on number of animals for spawning. Animals were always spawned in groups.

Spawned gametes were collected, counted, and measured under a compound microscope. Sperm was collected using a 1 mL disposable pipette and pooled into a 15 mL glass test tube on ice until fertilization. Eggs were collected and cleaned. Large debris was removed by pouring eggs through a 200 µm mesh sieve into a larger 50 µm sieve. The eggs were contained in this sieve and rinsed to eliminate excess organic matter using 1 micron, U.V. disinfected seawater at 22 °C prior to fertilization. Eggs were transferred into stagnant 1 L hatching beakers. Each cohort of egg was kept in a unique beaker to monitor egg quality (i.e. color, size, hatching

success, fertilization success, larval development). The pooled sperm previously collected was pipetted into the hatching beakers to achieve a sperm density of about 10^3-10^5 mL^{-1} during fertilization (Encena II *et al.* 1998; Hodgson *et al.* 2007). Gametes were gently mixed for 30 minutes using a spatula, which dispersed eggs throughout the water column during fertilization. Once fertilization was observed, eggs were rinsed in the 50 µm sieve to eliminate excess sperm and prevent polyspermy. Eggs were transferred back into hatching beakers filled with "culture medium" or 10 mg antibiotics (10,000 U/mL Penicillin-Streptomycin, Gibco, Sigma-Aldrich, St. Louis, MO, USA) per liter FSW.

The fecundity, fertilization, and hatching were recorded for each spawn event. It should be noted that spawned eggs of this species were observed to lack a chorion and jelly coat, which suggests shedding of this protective layer occurred as part of final oocyte maturation. This differs from other univalve species where hatching is defined by shedding of the chorion by the embryo prior to becoming free-swimming.

Fecundity = [total number of eggs spawned / gram of total body weight (including shell)]

Fertilization success (%) = [total number of eggs in cleavage (2-cell stage) / total number of eggs] × 100

Hatching success (%) = [total number of free-swimming larvae (trocophore stage) / total number of fertilized eggs (2-cell stage)] × 100

Hatched larvae were then passively transferred and stocked into 1 L larval rearing vessels at 10 larvae/mL culture medium. Larvae were left in stagnant larval rearing vessels until deemed competent for settlement.

4.2.4. Larval development

The observations of larval development at 22.26 ± 1.32 °C from unfertilized egg to metamorphosed postlarva were made using a compound microscope every 30 minutes. To collect samples, a 50 mL aliquot was pipetted from a single hatching beaker. From the aliquot, individual animals were randomly pipetted onto a microscope well plate. For direct observations of settlement, late veliger larvae were stocked into a series of petri dishes with a biofilm-seeded microscope slide. Slides were seeded with a non-axenic mix of naturally occurring diatoms in a flow-through aquarium under fluorescent light (100 µmol m^{-2} s^{-1}) for 14-21 days. The average biofilm density of n=3 slides (2.58 × 10^5 cells cm^{-2}) was quantified by scraping biofilm from both sides of the slide into deionized water, homogenizing on a mixer, and counting total number of cells using a hemocytometer. The duration of each developmental stage was recorded. Photographs of each stage were captured using a digital camera (WF200, AmScope, Irvine, CA, USA), and measurements done using imaging software program (AmScope 3.7). Late veliger larvae with developed sensory organs (i.e. eye spot, muscular foot, cephalic tentacle) that could retract into completely formed larval shells were negatively buoyant and considered competent to settle.

4.3. Results

4.3.1. Broodstock maturation

At the start of the study, broodstock had reduced gonads and sexes were predominantly unidentifiable. The monthly mean GSI value ranged from 3.8% to 14.6% during the study

period, with a significant (p<0.05) increase in GSI between October and February (Figure 4.1.). The relative gonad size was larger for males than females in all months (Figure 4.2). Direct observation of natural spawning occurred in broodstock tanks during the month of December, which indicated readiness to spawn. Only males were observed to spawn naturally (without hormone). The sex ratio of animals held in captivity was 3:1 female to male during this spawning season.

Figure 4.1. The gonadosomatic index (GSI) of captive reared *Cellana sandwicensis* from October to February. Bars are represented by mean ± standard deviation values. Monthly values with statistical differences (p<0.05) are indicated with asterisks (*).

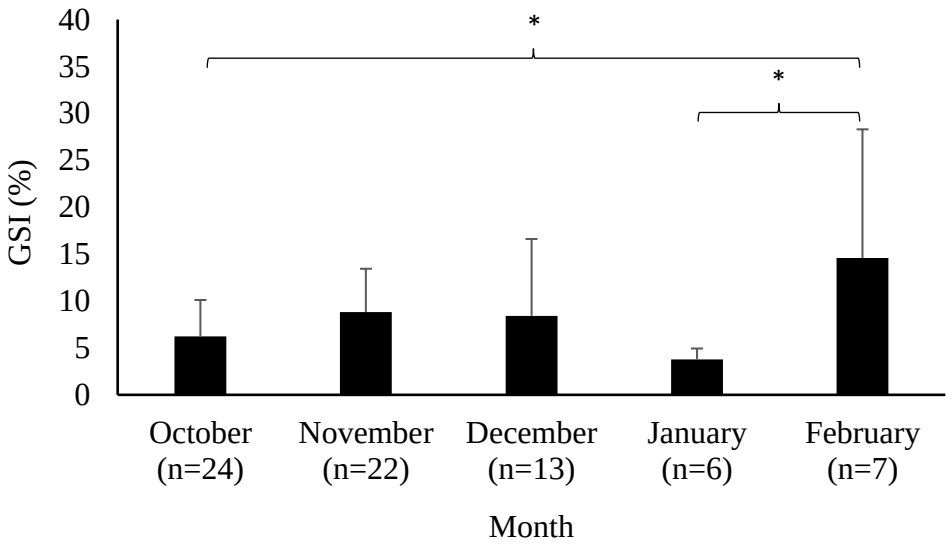
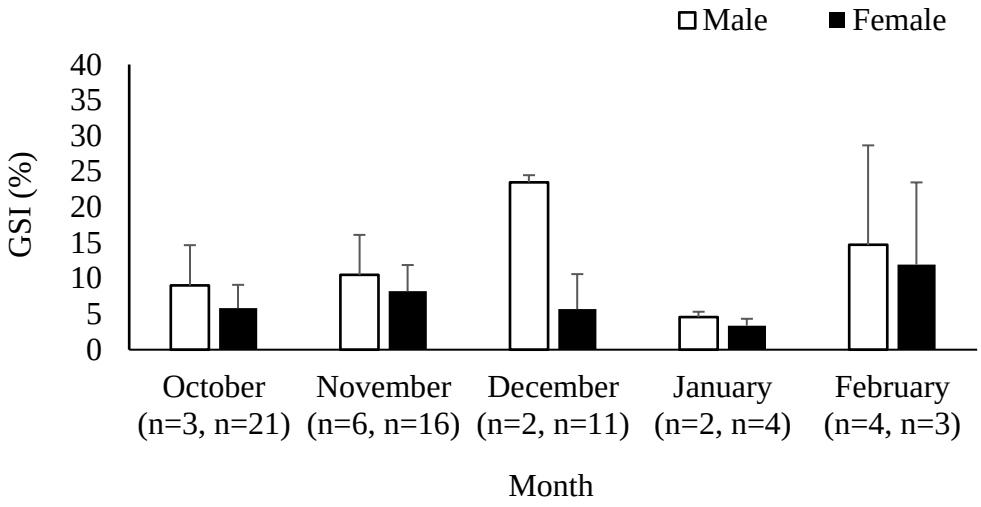


Figure 4.2. The gonadosomatic index (GSI) of male and female captive reared *Cellana sandwicensis* from October to February. Bars are represented by mean ± standard deviation values.



The GSI (n=66) was negatively correlated with both air temperature and water temperature (R^2 = -0.8309 and R^2 = -0.9321, respectively). These relationships between GSI and air temperature and GSI and water temperature were both significant (p = 0.049 and p = 0.006, respectively) during the study. We have excluded the month of January for this analysis because GSI in January appeared to be confounded with spawning observed in December.

4.3.2. Spawning

In each trial, all spawns occurred between around 00:00 hours (4 to 8 hours post-injection), with the exception of one immediate spawning individual upon insertion of the microliter syringe. There was no spawning in any control group. Stacking behaviours were observed in all beakers/buckets. Males always spawned first, as evident by release of sperm. The

use of hormones to control final maturation resulted in 10-13% spawning success. Fecundity (F) or total number of eggs per gram body weight for Trial 1 and Trial 2 was 3,964 and 3,846 eggs/g BW, respectively. For Trial 3, fecundity was 4,030 (female 1), 38,226 (female 2), and 24,135 (female 3) eggs/g BW, respectively. Fecundity was linearly correlated with total body weight (including shell) (regression equation: F=3007.8BW–14919; $R^2 = 0.7934$; $p < 0.05$). The best quality eggs from Trial 3 were reared with 35.4% survival to pre-settlement competency and stocked in the settlement petri dishes.

Table 4.2.

The reproductive traits of *Cellana sandwicensis* in three different spawn trials.

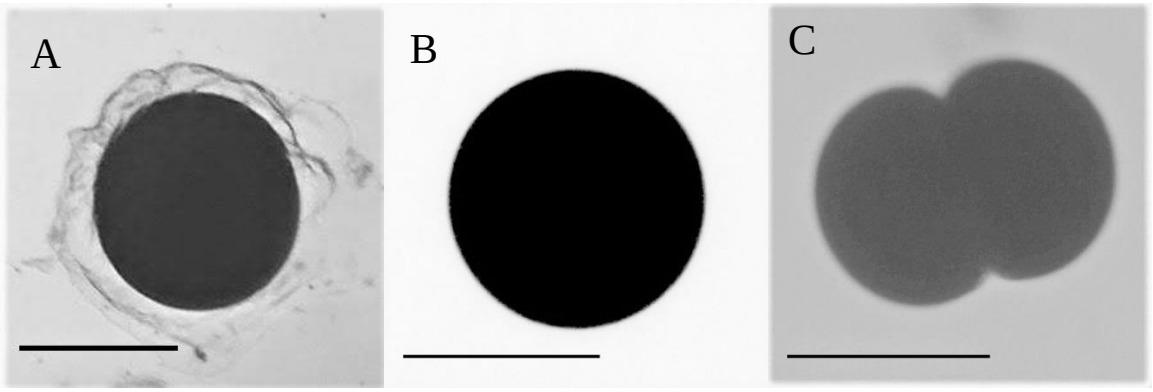
Trial	Hormone Injection(s) (ng g^{-1} BW)	*N*	No. of spawns	Spawn Success (%)	Total Eggs (10^3)	Total Sperm	Fert. Success (%)	Hatching Success (%)
1	Priming 250 + Resolving 500 sGnRHa	16	1M, 1F	13	41.5	4.20E+09	26	10
2	Priming 250 + Resolving 500 sGnRHa	37	3M, 1F	11	20.0	3.10E+08	28	29
3	Priming 250 + Resolving 500 sGnRHa	70	4M, 3F	10	21.6^1; 612^2; 300^3	-	90^1; 93^2; 85^3	10^1; 9^2; 85^3

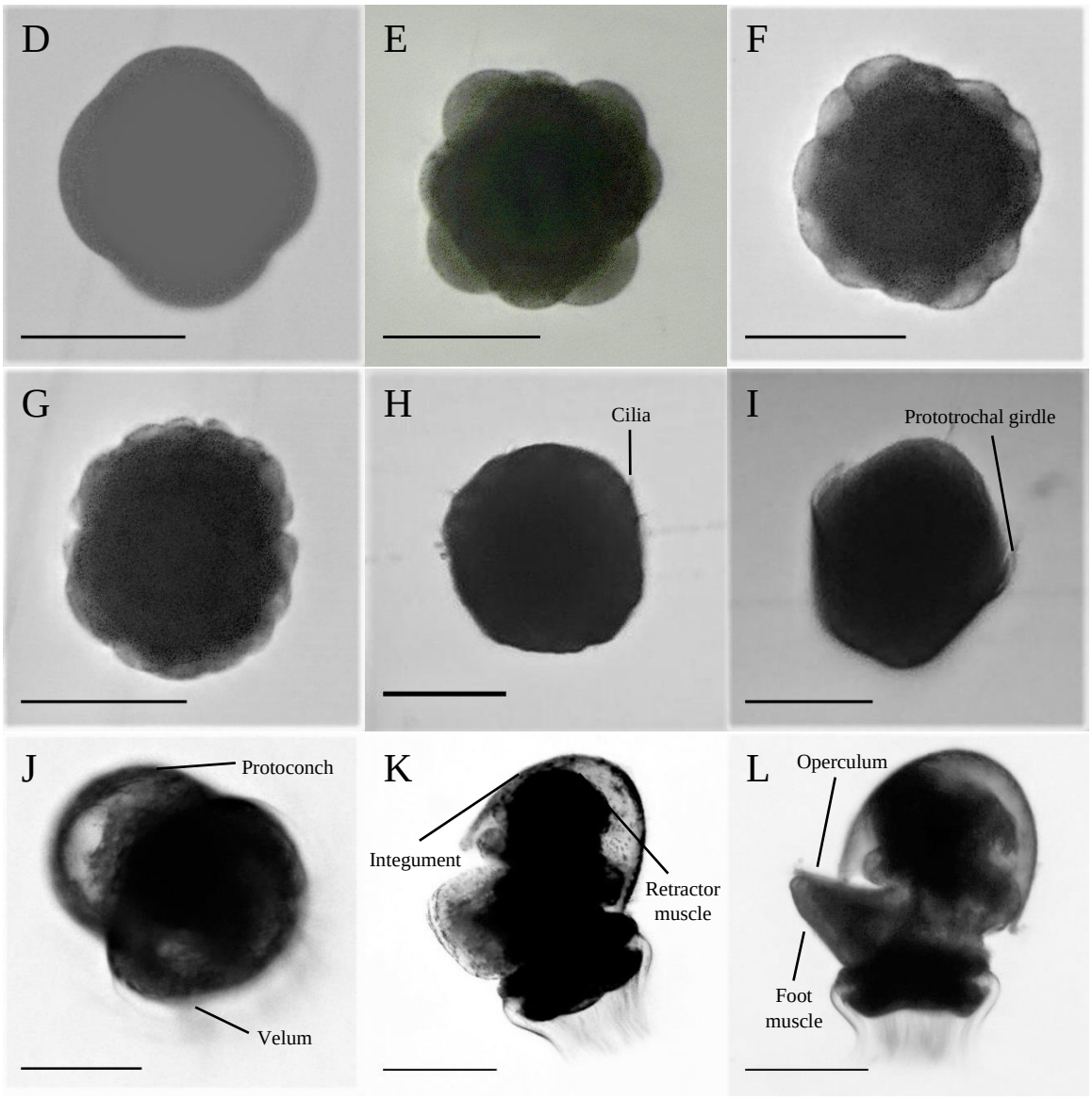
Superscripts (1-3) correspond to an individual female's spawn metrics.

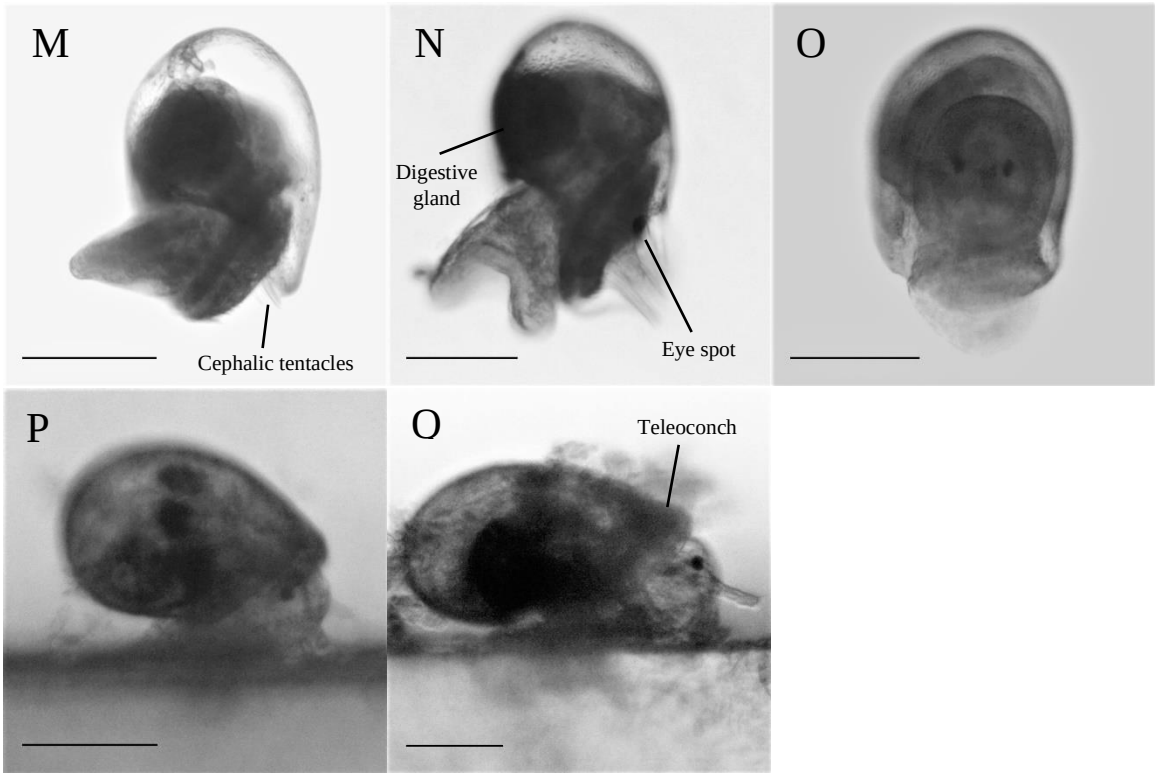
4.3.3. Larval development

The development of *C. sandwicensis* to metamorphosis occurred over a 5-day period (Figure 4.3.). After viable eggs (single female donor) and sperm (multiple male donors) were mixed, development across 16 major stages were identified (Table 4.3.). Due to rapid development, some key morphological changes were grouped into the same stage. The resolution of our observations was limited to every half hour.

Figure 4.3. The various stages of *Cellana sandwicensis* development from an oocyte to early postlarva. µm. (A) Stage 0, an oocyte with chorion present. (B) Stage 1, egg (lacking chorion), discharge of polar bodies. (C) Stage 2, 2-Cell. (D) Stage 3, 4-Cell. (E) Stage 4, 8-Cell to 16-Cell. (F) Stage 5, blastula. (G) Stage 6, gastrula. (H) Stage 7, development of cilia. (I) Stage 8, ciliated prototrochal girdle, trochophore larvae. (J) Stage 9, protoconch and velum. (K) Stage 10, integument and retractor muscles. (L) Stage 11, foot muscle, operculum and 180° torsion. (M) Stage 12, eye spot and appearance of cephalic tentacles. (N) Stage 13, digestive gland. (O) Stage 14, retraction into larval shell and passive settlement. (P) Stage 15, metamorphosis. (Q) Stage 16, development of teleoconch. Scale bar=100 micrometers.




D
E
F
G
H
Cilia
I
Prototrochal girdle
J
Protoconch
Velum
K
Integument
Retractor muscle
L
Operculum
Foot muscle



Spawned eggs with a mean diameter of 136.95 ± 5.02 µm were fertilized upon contact with sperm. Fertilization of the egg was determined by the discharge of the first and second polar bodies within 1 hour of exposure to sperm (Stage 1). Upon completion of the ciliated band, the trochophore larvae began to swim in circles and upwards in the water column from 9 to 10.5 hours. Late veliger larvae were considered competent for settlement, capable of retracting into their shell and passively settling which occurred from 48 to 72 hours. After passively settling, veliger larvae were observed to open their operculum and feel around the benthic environment with their muscular foot. If the environment was suitable for attachment (i.e. surfaces with biofilm), the larvae crept upright. When the environment was not suitable (i.e. surface void of biofilm) the larvae used their velum to swim. Metamorphosis or permanent attachment was defined by loss of the velum (pre-oral ciliated cells observed to be shed), extension of cephalic

tentacles, and cilia formation on the foot that occurred as soon as 36 hours post-attachment (Stage 15). Finally, the teleoconch was observed to grow as an extension of the protoconch after 24 hours post-metamorphosis (Stage 16). This shell grew faster than the protoconch and in an asymmetrical fashion over the cephalic region of the postlarval animal, which resulted in body elongation (Table 4.4.).

Table 4.3. The embryogenesis and larval development of *Cellana sandwicensis* at 22 °C.

Stage	Time (hours)
Fertilization	0.0
Discharge of polar bodies	0.0-1.0
2-Cell	1.0-2.0
4-Cell	2.0-2.5
8-Cell to 16-Cell	2.5-3.0
Blastula	3.0-3.5
Gastrula	3.5-4.0
Cilia forming prototrochal girdle	4.0-7.5
Ciliated prototrochal girdle	7.5-9.0
Trochophore	9.0-10.5
Larval shell secretion (protoconch), velum formation	10.5-12.5
Integument and retractor muscle	12.5-16.0
Foot muscle, operculum, digestive gland, 180° torsion	16.0-24.0
Eye Spot and appearance of cephalic tentacle	24.0-36.0
Digestive Gland	36.0-64.0
Passive Settlement	48.0-72.0
Metamorphosis (Loss of velum, crawling up-right, ciliated foot, cephalic tentacle)	82.0-138.0
Adult shell (teleoconch)	106.0-130.0

Table 4.4. Measurements (µm) of *Cellana sandwicensis* during key stages of development (n=6). Length=L, Width=W.

	Measurements							
	Trochophore		Early Veliger		Late Veliger		Postlarva	
	W	L	W	L	W	L	W	L
Mean	148.16	160.09	168.21	181.85	161.97	218.84	161.98	253.65
Stdev	3.43	8.12	15.15	19.01	14.22	15.88	13.17	11.76

4.4. Discussion

4.4.1. Maturation

Maturation of yellowfoot limpet *C. sandwicensis* followed similar patterns previously observed in both the laboratory (Hua and Ako 2014) and natural environment (Kay *et al.* 1982), which indicates rearing conditions were acceptable.

The maturation cycle of captive broodstock appeared to be temperature dependent. Sternberg *et al.* (2010) reported that gastropod reproduction is predominantly influenced by, aside from nutrition, temperature and photoperiod regimes. This relationship has been documented to be similar for other wild limpets *C. grata* (Liu 1994) and *C. nigrolineata* (Catalan and Yamamoto 1993), where reproductive cycles followed seasonal sea temperature changes. We suggest that temperature may play a role in gametogenesis that must be examined further – by itself or in combination with photoperiod – to support year-round captive maturation.

As far as evaluating final maturation in aquaculture, GSI is a standard measurement. However, there was substantial deviation from the mean GSI for each group across spawning season, which poses some limitations. By analyzing GSI by sex (Figure 4.2.) we can explain

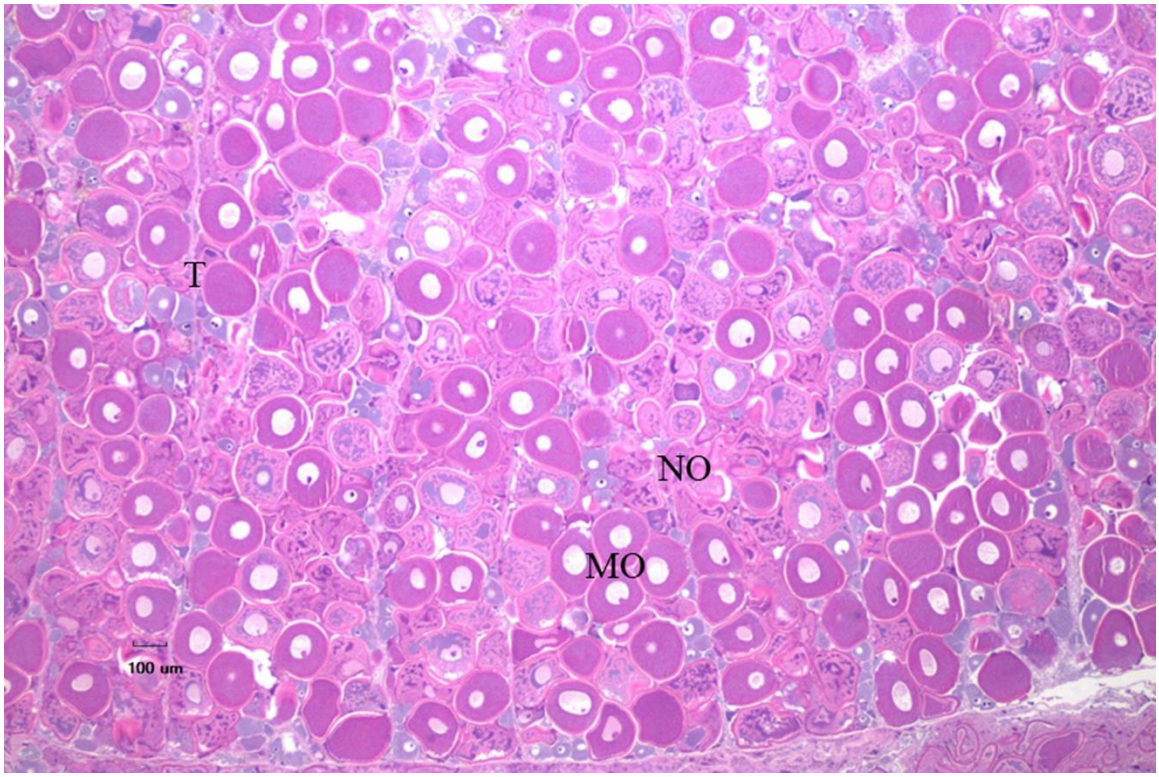
some of this deviation, however, we could not perform statistical analysis due to low n-values. Peak mean GSI for males (December, 23.49%) occurred earlier and was 2-fold greater than peak mean GSI for females (February, 11.93%), which has been previously reported to be the case for other patellid limpets (Morriconi 1999; Morais *et al.* 2003) as well as wild Hawaiian limpets (Kay *et al.* 1982). Moreover, the substantial deviation from mean GSI may also be due to asynchronous gametogenesis, a biological phenomenon for the tropical mollusc *Haliotis varia*; however, temporal histological examination is required to make that type of interpretation (Najmudeen 2007). We recommend using a combination of GSI and histology to effectively assess maturation of limpets in future experiments.

4.4.2. Spawning

The peptide hormone GnRH is known to play a role in reproduction for across both vertebrate (Cheng and Leung 2005) and invertebrate taxa (Nuurai *et al.* 2016). In this study, we applied sGnRHa to synchronize spawning events for captive matured *C. sandwicensis*. The spawning success was low (10 to 13%) across three separate trials, which may have been a result of the particular hormone level or that only a fraction of the total population was in final maturation at any given time. Based on high standard deviations from mean GSI values in February—when spawning trials were initiated—inconsistent gonad development may have reduced GnRH's efficacy. Additionally, histology of an ovary sampled prior to conducting spawning trials showed necrotic oocytes – previously described by Morriconi (1999) to be a characteristic of a "totally spawned stage" (Figure 4.4.); and perhaps limpets should have been spawned earlier – a timing issue. The overall efficacy of this spawning method, as measured by spawning success, must

therefore be further examined with respect to GSI, stage of oocyte development, and time to improve hatchery success.

Figure 4.4. The histological examination of ovary tissue from *Cellana sandwicensis* sampled in February at 40x magnification. The ovary contains clusters of both mature oocytes (MO) and necrotic oocytes (NO). Trabeculae are represented as (T). Scale bar=100 micrometers.



During spawning trials, sperm was collected first and used as a pheromone to heighten spawning activity. A group of molluscan peptides have been characterized as pheromones, which induce the release of eggs, attraction of sperm to fertilize eggs, or aggregation during spawning events (Susswein & Nagle 2004). For *C. sandwicensis*, the inoculation of sperm into beakers was found to trigger or enhance "stacking behaviour" during reproduction, a similar phenomenon exhibited by other molluscs such as the Antarctic limpet *Nacella concinna* (Picken & Allan 1983)

and Pinto abalone *Haliotis kamtschatkana* Jonas (Stekoll & Shirley 1993). This is the first reported case (in wild or captivity) for Hawaiian limpets.

Cellana sandwicensis exhibit size-dependent fecundity, which is common for many broadcast-spawning benthic invertebrates (Llodra 2002). We chose to measure fecundity on a weight-basis because limpet fecundity was most significantly correlated to total weight. Given this information, size-selecting larger broodstock would most likely improve hatchery output metrics. By contrast, using smaller broodstock, especially ones in their first reproductive semester may result in subpar spawn qualities and reduced production. The effect of broodstock size on spawn quality and survivorship should be considered to maximize hatchery effort, as well as to make informed wild fisheries management decisions pertaining to size-specific regulations (Hawai'i State Law prohibits *Cellana spp.* <31.8 mm shell length; Department of Land and Natural Resources Section 13-92-1).

4.4.3. Larval development

Larval development was documented across 16 major stages to the postlarva stage (Table 4.3., Figure 4.3.). These stages may have multiple morphological changes if occurrence was simultaneous. The timeline of these stages is reported with accuracy to the half-hour, which is due to the variable development between individual larvae of the same egg cohort. The rate of development to metamorphic competence (ca. 3.5 days) was much more rapid than expected. For *C. exarata*, which inhabit higher regions of the intertidal, larvae reach metamorphic competence between 5- and 10-days post-fertilization (dpf), and larval duration recorded to 18 days (Corpuz

1981). The short larval period of both Hawaiian limpets is a concern for re-seeding of overharvested or depleted populations.

These larvae were reared in clear water, which confirms that *C. sandwicensis* are lecithotrophic and do not require planktonic food sources (Bird *et al.* 2011). Instead, their larval energy sources are likely derived from internal yolk-sac (maternal) and exogenous dissolved organic matter (Jaeckle & Manahan 1989). Egg color, which varied from dark yellow to brown, was derived from the yolk-sac containing parental pigments. The pigment retention of eggs may vary based on broodstock diet and associated pigments as found to be the case for sea urchins (Carboni *et al.* 2015). Moreover, immature ovaries were observed to be a much lighter hue compared to mature ovaries (loose oocytes), and we suspect there to have been differences in yolk granule densities between egg cohorts, possibly contributing to differences in egg quality (Trial 4.3., Table 4.2.).

The development of *C. sandwicensis* was nearly identical to various species of gastropods through the trochophore stage (Balkhair *et al.* 2016). Cell division after the 4-cell stage was too rapid to include each division as unique stages, henceforth Stage 4 refers to both the 8-cell and 16-cell division. The vertical suspension of trochophore larvae from sister taxa *C. exarata* was previously considered to be a function of their negative geotaxis (Corpuz 1981). However, we observed yellowfoot limpet larvae to move towards any applied light source, making them positively phototactic. Pre-torsional larvae were observed to undergo monophasic 180° torsion process, where the larval retractor muscles contracted over a few minutes. This rotation was supported by simultaneous pedal muscular development but preceded the development of the operculum and completion of the larval shell.

Retraction and passive settlement were observed only after torsion was completed and sensory organs developed (i.e. muscular foot, eye spots, and cephalic tentacles). During retraction, the operculum appeared to function as a trap-like door during passive settlement, which was shed prior to creeping (up-right attachment). Unlike other marine invertebrates, the descent to the benthos for *C. sandwicensis* is by passive settlement, which we observed to occur in clear water vessels void of chemical cues. However, more work is required to understand influencers of settlement with respect to attachment and metamorphosis.

4.5. Conclusion

Yellowfoot limpet (*Cellana sandwicensis*) remains a prospective candidate aquaculture species due to high fecundity, extremely short larval duration, and non-feeding larval phase. In this study, practical methods for maturation, spawning, and larval rearing have been devised for aquaculture. The first descriptions of spawning and early life-history give essential information that can be used in aquaculture and fisheries management. Future research will focus on settlement and grow-out aspects for this species.

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Chapter 5

Gonadotropin Releasing Hormone-like peptide is not an acute spawn-inducer for Prosobranch Limpets (*Cellana spp.)*

Abstract

Multi-functional group of gonadorotropin releasing hormone (GnRH) forms have been identified in nearly all metazoans, including invertebrate molluscs. To date, few GnRH-like molecules (i.e. oct-GnRH and ap-GnRH) have been characterized for both structure and function; and until recent reviews, GnRH-like neuropeptides were categorized with chordate forms that are associated with reproduction. In general, reproductive endocrine systems of molluscs are not well-understood, and research investigating pathways of key neuromodulators and neurohormones has been of interest. Putative limpet GnRH-like peptides have been identified in two Prosobranch species *Lottia gigantea* and *Patella caerulea*, and characterization of its function has never been examined. Given recent studies describing s-GnRH (salmon) analogue ability to stimulate reproduction in another Prosobranch, *Cellana sandwicensis*, authors aimed to test spawn-induction effects of ol-GnRH (owl-limpet) administered as both hormone and pheromone in *Cellana spp*. To derive the bioactive ol-GnRH, we used phylogenetic analysis of data-mined GnRH primary structures. The resulting tree suggested molluscan GnRH-like to be monophyletic, and supported a pair of identical limpet GnRH-like sequences. We synthesized ol-GnRH based on these homologous undecapeptides amino acid sequence with an *N*-terminal pyroglutamic acid residue and *C*-terminal amidated glycine – essential post-translational modifications for GnRH bioactivity. In three separate experiments, peptide was administered either via intramuscular injection into the gonadal cavity

or administered as a pheromone in sterilized seawater, which showed lowest rate of degradation based on zero-order kinetics. Resulting spawns in control groups for both bioassays suggest that s-GnRH and ol-GnRH do not have spawn-induction effects on Prosobranch limpets *Cellana* ($p>0.05$). Spawns appeared to occur sporadically between hours 00:57 and 06:54, and spawn success (%) remained relatively low for all experimental treatments where limpets could not interact with each other. Despite these partially contradictory results, null-findings were in agreement with recent paradigm-shifting discussion on GnRH-like form and function. Authors further consider that putative ol-GnRH may stimulate non-reproductive responses similar to those characterized for oct-GnRH (Octopus) and ap-GnRH (Aplysia), without discounting other possible functional/modal pathways. This study represents the first *in-vivo* study of putative ol-GnRH function using a Prosobranch limpet model, and spurs further research on GnRH-like molecular characterization. Moreover, research on endocrinology of commercially important molluscs will surely impact both wild fisheries and aquaculture industries.

5.1. Introduction

Gonadotropin-releasing Hormone (GnRH) is present across all studied metazoan taxa. The nomenclature and classification of various forms of this molecular group has been a recent topic of debate (Tsai 2018; Zandawala *et al.* 2018), especially after isolation of the first GnRH-like structures from Octopus *Octopus vulgaris* (Iwakoshi *et al.* 2002). The focus has since shifted from pituitary GnRH as the driver of hypothalamus-pituitary-gonadal axis (HPG axis) in chordates to characterizing other non-pituitary forms, including molluscan forms – a group of undecapeptides and dodecapeptides with an *N*-terminal pyroglutamyl and *C*-terminal amidated glycine (Tsai & Zhang 2008). Recently, studies have elucidated these putative GnRH-like molecules in various

groups using -omics approaches, spurring further investigation of functional conservation and diversification (Minakata & Tstsui, 2016).

For Gastropods, GnRH-like isoforms appear to maintain a mixture of functions of which appear to support reproduction both directly and indirectly. In sea hare *Aplysia californica*, detection of GnRH-like molecules in the hemolymph, CNS, and osphradium (olfactory organ) suggests these peptides play multiple roles in reproduction – acting as both hormones and neurotransmitters/neuromodulators (Zhang *et al* 2000; Tsai *et al.* 2003). When synthetic ap-GnRH was injected into *A. californica*, it failed to effect various measures of gonad development and stimulate egg-laying, however, stimulated behavioral responses like parapodial opening, inhibition of feeding, and promotion of substrate attachment (Tsai *et al.* 2010). Similarly, another synthetic GnRH-like molecule characterized from abalone *Haliotis asinina* appears effective in stimulating oocyte proliferation during reproduction, but has yet to be examined as a spawn-inducer (Nuurai, 2016). Interestingly enough, Nuurai *et al.* (2010) studied the effect of other isoforms as spawn-inducers on *H. asinina*, and observed a dose-dependent response to chordate GnRH, but a negative response to a molluscan GnRH.

There have also been two Prosobrach limpets (*Lottia gigantea* and *Patella careulea*) identified to have a putative GnRH-like neuropeptide encoded in their genome. The prohormone amino acid sequences are identical, and therefore researchers have postulated this isoform is well-conserved by structure and function (Veenstra 2010; De Lista *et al.* 2013). The characterization of these putative limpet GnRH-like molecules is imperative to the reproductive endocrinology of key commercial and aquaculture species (Mau & Jha 2018).

In a previous *in-vivo* study, Hua and Ako (2013) administered a series of intramuscular injection of salmon GnRH analogue at 250 ng/g BW to yellowfoot limpet *Cellana sandwicensis*,

which increased the gonadosomatic index (GSI) by 31% compared to a saline-injected, control group. Although recent studies by Hua and Ako (2013) and Mau *et al.* (2018) have both reported the possibility for this hormone to stimulate spawning in ripe limpets, limited experimental design warrants further investigation of any spawn-inducing effect.

In the current study, we examined the effect of synthetic GnRH analogues – salmon GnRH (s-GnRH) and owl limpet GnRH (ol-GnRH) – on acute spawning in two Patellid limpet species, *C. exarata* and *C. sandwicensis*. We also tested two administration methods, hormone injection and pheromone exposure, in side-by-side bioassays across three independent experiments to determine if these GnRH isoforms have multiple modalities.

5.2. Materials and Methods

5.2.1. Molecular Phylogenetic Analysis

Gonadotropin releasing hormone (GnRH) peptide sequences were obtained from the National Center for Biotechnology Information website (NCBI). Amino acid sequences of predicted structures were identified using BLASTx algorithm function in NCBI's database. Sequences were aligned by ClustalW multiple sequence alignment, and a Maximum Likelihood phylogenetic tree based on Poisson correction model was constructed in MEGA7 (Kumar *et al.* 2016). The phylogenetic tree was rooted with Molluscan Insulin (GenBank Accession Number, NP_001191615.1), and reliability was assessed with bootstrapping using 10,000 replicates. For this study, the orthologs (*species*, GenBank Accession Number – *Haliotis asinine* (AKR13998.1), *Haliotis laevigata* (AKR13997.1), *Haliotis discus hannai* (ARE3028.1), *Aplysia californica* 1

(NP_001191482.1), *Aplysia californica* 2, *Patella caerulea*, *Lottia gigantia*, *Sepiella japonica* (ALS92801.1), *Octopus vulgaris* (BAB86782.1), *Ruditapes philippinarum* (A0A0A7DNP6.1), *Crassostrea gigas* (NP_001292223.1), *Crassostrea virginica* (XP_022319572.1), *Petromyzon marinus* 1 (AAF78456.1), *Petromyzon marinus* 2 (ABE66462.1), *Petromyzon marinus* 3(ABE66462.1), *Homo sapiens* 1 (NP_000816.4), *Homo sapiens* 2 (AAC02981.1), *Gallus gallus* 1 (NP_001074346.1), *Gallus gallus* 2 (BAE80717.1), *Squalus acanthias* (P27429.1), *Oncorhynchus mykiss* (AAD43461.1), *Oncorchynchus keta* (P69105.1), *Ciona intestinalis* (NP_001027799.2), *Sparus aurata* (P51919.1), *Oryzias latipes* (NP_001098169.1), *Clupea harengus pallasi* (P81749.1), *Strongylocentrotus purpuratus* (XP_800179.1) are classified by five neurohormone types (GnRH I to GnRH V) according to primary structure and their conserved amino acids.

5.2.2. Peptide Synthesis and Profile

The proposed, bioactive sequence of owl limpet GnRH analogue [pGlu-His-Tyr-His-Phe-Ser-Asn-Gly-Trp-Lys-Ser-NH_2] or ol-GnRH was assembled manually using Fmoc chemistry, and cleaved, as described by Kapono *et al.* (2013) (see Appendix A).

The crude cleaved peptide was purified by semi-preparative C_{18} RP-HPLC/UV fractionation using a mobile phase, Solvent B (MeCN; 90/10 MeCN /0.08% v/v aq. TFA), against Solvent A (0.1% v/v aqueous Trifluoroacetic acid) at a flow rate of 1 mL/min for 80 min. This was undertaken by employing a Waters Alliance 2695 System interfaced with a Waters 996 Photodiode Array Detector (PDA). Chromatographic data was acquired, integrated and analyzed using Waters Empower Software (V.2).

Fractions were chemically profiled using an RP-HPLC/UV method modified from Chun *et al.* (2012). Manually collected fraction samples were analyzed by capillary bore HPLC (250 x 1 mm, 5 µm; 300Å; Phenomenex, Torrance, USA) using a linear gradient 1% /min with a flow rate of 0.1 mL/min for 55 min. The target fractions were pooled and lyophilized, and stored at -20°C until required. Electrospray Ionization Mass Spectrometry (ESI-MS) was performed on purified peptide according to Chun *et al.* (2012) using AB/MDS-Sciex API 3000 triple quadrupole mass spec-trometer (Thorn hill, Ontario, Canada).

5.2.3. Peptide Stability

An assay was performed to determine the stability of ol-GnRH peptide in filtered seawater (FSW) and sterilized seawater (SSW) at 25°C and 37°C. To prepare FSW, natural surface seawater (salinity 35 ppt, pH 8.3) was collected 12 hours prior to the start of experiments at The Waikiki Aquarium, Honolulu, Hawai'i, and filtered through a 1 µm mesh bag. To prepare SSW, the previously filtered seawater was sterilized by autoclave in a 121 ℃ oven for 50 min. These seawater treatments were both stored in sterile, closed 1L Nalgene containers at ambient room temperature until being used.

For all treatments (FSW25, FSW37, SSW25, SSW37), a stock solution was made by dissolving peptide into a carrier, 0.01M phosphate buffered saline (0.01M PBS; pH 7.4; Sigma Aldrich, USA), and further diluted by FSW and SSW to achieve starting concentration of 167 µg/mL, respectively. Each treatment started with a volume of 15 mL stock solution previously transferred into a 15 mL polypropylene Falcon tube (Thermo Fisher Scientific, USA).

Experimental solutions were maintained in a dark, temperature-controlled air-conditioned laboratory at 25 °C or placed in a heating dry block set to 37 °C for the duration of the experiment.

At each time point, 0, 1, 2, 4, 8, 24, 48 hours, stock solutions were mixed and sampled in triplicate by transferring 100 µL into a sterile 2 mL Eppendorf tube (Eppendorf, USA). Each replicate sample was lyophilized and stored at -20 °C until being analyzed.

A standard solution was prepared by dissolving ol-GnRH in Solvent A. For each set of RP-HPLC/UV analyses, a standard curve was obtained using linear regression analysis of the standard solution injected at 5, 7, 10, 12, 15 µL.

Prior to chemical analysis, each sample was prepared by resuspending the freeze-dried material in Solvent A, and centrifuging at 12,000 RPM for 1 min.

The concentrations of each sample were quantified using the area under the target peak, and averaged to calculate percent degradation (%):

% degradation = [1-(sample concentration/starting material concentration)] x 100

Degradation kinetics of GnRH under various conditions were monitored across 48 hours. The Coefficient of Variance ($\sigma x/\bar{x}$) ranged from 2.7-11.9% for time-point triplicates. The kinetics were determined by plotting data assuming zero, first, and second order kinetics, and evaluating goodness-of-fit by the correlation coefficient (R^2). Half-life was determined using zeroth-order reactions:

$$\mathbf{T_{1/2}} = [A]/2k$$

Where A is the concentration of peptide in mol/L and k is the rate constant (slope).

5.2.4. Experimental Animals

Mature animals were collected from the rocky intertidal zone prior to each experiment. The blackfoot limpet, *Cellana exarata*, were collected from an undisclosed location on the windward shoreline of Big Island, Hawai'i (Location A). The yellowfoot limpet, *Cellana sandwicensis*, were collected from an undisclosed location on the windward side of Oahu, Hawai'i. Each group was acclimated for two weeks in an aquaculture laboratory following Mau *et al.* 2018. Mortalities from collection/acclimation (n=7-12) were dissected for gonadosomatic index (GSI) and visual assessment of maturation status prior to use in spawn induction bioassays. Sexing of live animals was not possible.

5.2.5. Spawn Induction Bioassays

Spawn induction bioassays were performed in three separate experiments on 1/26/18, 12/11/18, and 1/18/19 (Table 5.1.). For all bioassays, the administration of salmon GnRH (s-GnRHa, Syndel Chemicals, USA) and owl limpet GnRH (ol-GnRH) isoforms by hormone injection and by pheromone exposure were tested side-by-side with a unique sub-set of animals. Each experiment started at 20:00h and ended at 08:00h the following morning – a 12 hour period based around *Cellana spp.* natural spawning in captivity (Pers. Obs.). Spawn activity was observed every 30-60 min. and recorded. Spawning is reported as presence/absence (+/-) of egg and sperm. Gametes were checked for viability by fertilization following Mau *et al.* (2018). After each experiment, animals were monitored for 14 days for mortality. Fisher's exact test was performed to determine if treatment and spawn frequencies were independent.

Table 5.1. Experimental conditions and associated linear equation, rate constant (k), half-life ($t_{1/2}$), and correlation coefficient (R^2) obtained from zero-order kinetics of the degradation of synthetic ol-GnRH peptide in filtered seawater (FSW) and sterile seawater (SSW).

Experimental Condition	Zero Order Kinetic Equations	k (M/s)	$t_{1/2}$ (h)	R^2
SSW25	y = -3.26261E-11x + 7.85024E-05	3.26E-11	295	0.978
FSW25	y = -4.88528E-10x + 6.96018E-05	4.89E-10	20	0.978
SSW37	y = -1.08552E-10x + 6.62765E-05	1.09E-10	84	0.940
FSW37	y = -2.88347E-10x + 6.98005E-05	2.88E-10	34	0.995

5.2.5.1. Hormone Injection

For hormone injection bioassays, a two-step injection method was applied. A single combination treatment (250, 500 ng g BW) was administered into the coelomic cavity via the muscular foot using a 26-gauge microliter syringe (Hamilton, USA). The injections were offered 24 hour apart, and volumes ranged from 5-10 µL per individual for priming and resolving doses. The control groups (0.01 M PBS) were administered with equivalent volumes on a per-body-weight basis. Animals were selected at random for each treatment, and monitored between priming and resolving injections in their respective group holding tank. At the start of the experiment, animals were stocked into spawning beakers previously filled with 1 µm, UV-treated seawater.

5.2.5.2 Pheromone Exposure

For pheromone bioassays, peptide solutions were diluted with 1 µm-filtered, UV-treated seawater to achieve the following concentrations: low (0.05 mg/L), medium (0.25 mg/L), and high (1.25

mg/L). The control solution was void of peptide. Animals were selected at random for each treatment, and stocked into spawning beakers previously filled with these solutions.

5.2.5.3 Experimental Design

5.2.5.3.1. Experiment 1

The blackfoot limpet *Cellana exarata* (n=71) mean SL 39.69±3.86 mm and weight 8.49±2.65 g were stocked into 1L polypropylene beakers filled to 500 mL. These beakers were lined with a turf ring just above the water level to limit vertical migration, and heavily aerated. For this experiment, animals were nested in groups of 4-5 individuals, and supplied heavy aeration. There was n=3 replicate groups per treatment in the hormone injection bioassay. The pheromone bioassay lacked technical replication, and serves as preliminary testing for this administration method.

5.2.5.3.2. Experiment 2 & 3

The yellowfoot limpet *Cellana sandwicensis* in Experiment 2 (n=60) mean SL 42.34±4.17 mm and weight 13.09±4.23 g and Experiment 3 (n=56) mean SL 42.54±4.66 mm and weight 12.79±3.16 g was stocked into 200 mL beakers filled to 150 mL (accommodating for volume displacement by animal). These beakers were covered with a plastic grate to limit vertical migration and with stagnant conditions. For these independent, side-by-side bioassays, animals were stocked individually with n=6 and n=8 replicate limpets per treatment, respectively.

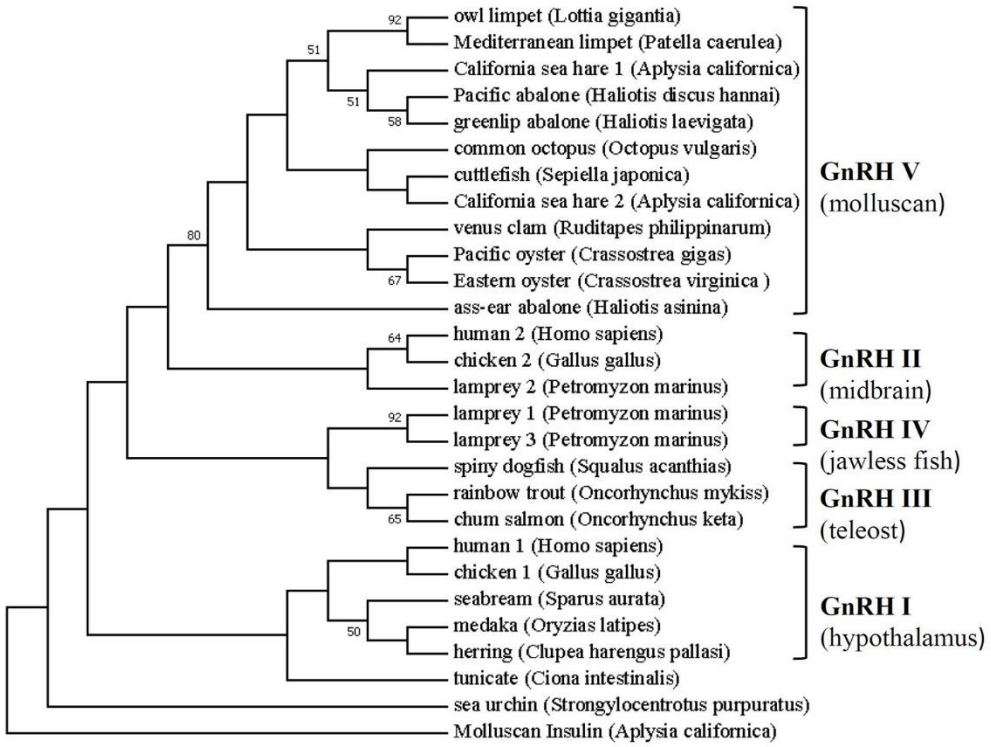
It should be noted that experimental conditions (aeration vs. stagnation and group vs. individual) were tested prior to Experiment 2 and Experiment 3, and found to have no significant effect on spawn induction for *C. sandwicensis* that would confound results of this study (unpublished).

5.3. Results

5.3.1. GnRH Primary Structures

An incomprehensive list of amino acid (aa) sequences derived from primary literature sources and/or NCBI's database were grouped based on their primary structures (not functional conservation/diversion). In Figure 1., GnRH I were hypothalamic/pituitary forms, GnRH II were mesencephalic/midbrain forms, GnRH II were teleost fish forms, GnRH IV were jawless fish forms, and GnRH V were molluscan forms. GnRH V, a monophyletic group, was applied to all 11-12 primary structures with two additional aa inserted at position 2 and 3. Owl limpet (ol-GnRH) and Mediterranean limpet (p-GnRH) sequences were identical, and divergence from a common ancestor was well-supported with a bootstrap value of 92. The shared primary aa sequence for this clade was selected for this study, and synthesized with the predicted structural modifications: *N*-terminal pyroglutamic acid and *C*-terminal amide.

Figure 5.1. Phylogenetic analysis of GnRH peptides from vertebrate and invertebrate taxa using Maximum Likelihood method in MEGA7. The phylogenetic tree was constructed using ClustalW multiple-sequence alignment of amino acid sequences, and is rooted by Molluscan Insulin *Aplysia californica* (outgroup). A bootstrap test was performed using 1,000 replicates, and confidence values (%) are reported on branch nodes. Peptides are classified by primary structure into five groups (GnRH I to GnRH V).



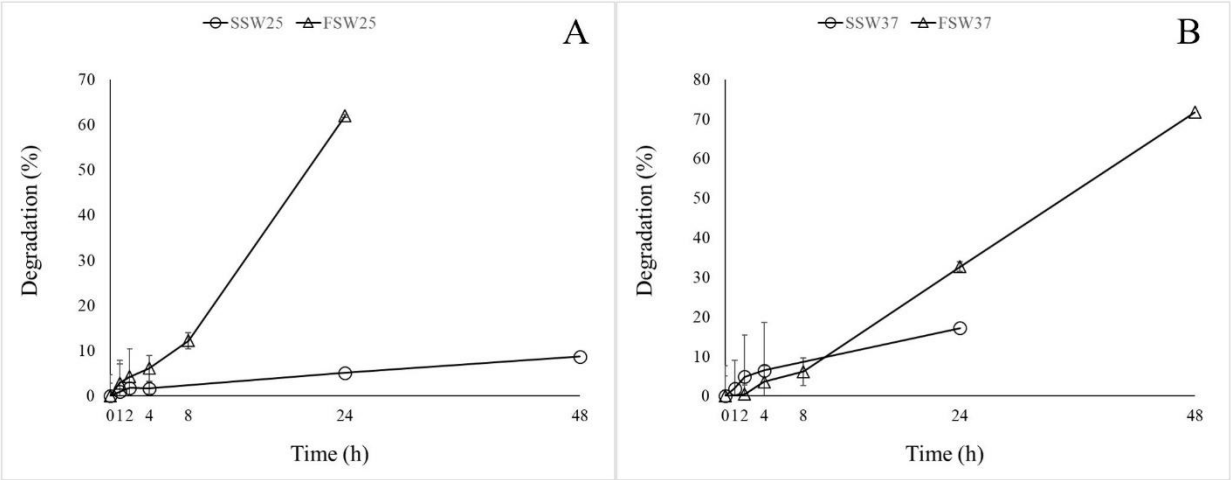
5.3.2. Synthetic ol-GnRH characterization

Synthetic ol-GnRH peptide was soluble in seawater. The RP-HPLC/UV chromatogram of ol-GnRH at 214 nm showed a retention time of 30.7 mins. Further analysis by ESI-MS showed an *m/z* of 687.7 Da as a doubly charged molecular ion ($[M+2H]^{2+}$), which agreed with predicted molecular weight of 1372 Da (see supplemental data).

Figure 5.2 A/B are linear plots of peptide degradation (%) in filtered and sterile seawater at ambient temperature (25° C) and elevated temperature (37° C) conditions. From these results,

the degradation of peptide was considered to be zero-order reaction. The rate constant (k) (Table 5.1.) increased with temperature in sterile seawater, however, was highest overall in filtered seawater at ambient conditions. Degradation was reduced for respective sterilized seawater treatments compared to filtered seawater.

Figure 5.2. Peptide degradation curves analyzed by RP-HPLC/UV (n=3). Error bars represent standard deviation of the mean. A) The degradation (%) of ol-GnRH in sterile seawater (SSW)(〇) and 1 µm filtered seawater (FSW)(△) incubated at 25°C. B) The degradation (%) of ol-GnRH in sterile seawater (SSW)(〇) and 1 µm filtered seawater (FSW)(△) incubated at 37°C.



5.3.3. Spawn Induction Experiments

A series of independent, side-by-side bioassays were summarized in Tables 5.2. and 5.3. Spawns were collected from every treatment (including controls) between 00:57h and 06:54h. Overall, there was no effect of hormone/pheromone treatments on acute spawn-induction of limpets ($p > 0.05$).

In Exp. 1, *C. exarata* were spawned in groups, thus making it difficult to track spawns. In Exp. 2 and Exp. 3, spawn success across treatments ranged from 0-25% for *C. sandwicensis*. For

spawning females (n=3), egg diameters were consistent in size, 147.52±2.11 µm (± SEM), but fecundity ranged from 3714 to 33793 eggs g^{-1} BW^{-1}. All spawned eggs were viable, and developed into competent veliger larvae by 24 hours post-fertilization.

When comparing administration methods, overall spawn success was not different between pheromone exposure and hormone injection bioassays. There was, however, a 2-fold increase in mortality for animals administered hormone injection (22-39%) compared to pheromone exposure (12-23%) following each experiment.

5.4. Discussion

GnRH neuropeptides, including the putative owl limpet GnRH-like peptide were tested in Prosobranch limpets (*Cellana* spp.).

In the preliminary experiment (Table 5.2.), pre-spawn behaviors such as aggregation and stacking were observed across both bioassays. These recently described tactile and/or chemical forms of communication, theorized to be critical for successful reproduction in broadcast-spawning limpets, may have introduced confounding stimulatory effects (Mau *et al.* 2018). Also, for these grouped bioassays, individual spawns could not be tracked with confidence, which limited collection of data. Despite these issues, egg and sperm were observed in all replicate beakers administered ol-GnRH hormone injections, and spawn frequencies were estimated as high as 50-100%. This spawning success was higher than findings previously reported for *C. exarata*, and provoked further testing to determine if GnRH forms indeed activate spawning.

Table 5.2. A preliminary side-by-side bioassay investigating the effect of GnRH type III (sGnRH) and GnRH type V (olGnRH) on spawn induction of blackfoot limpets (*Cellana exarata*). Wild, ripe animals ($N_{Exp.1}$=71) were collected from a rocky intertidal shoreline and spawned in groups of 4-5 limpets supplied with heavy aeration. Sex ratio (female to male) and gonadosomatic index (mean ± SD%) were determined prior to the experiment. The hormone injection bioassay treatments were replicated in groups (n=3).

Experiment	Date	Location	Species	Sex Ratio (f/m)	GSI (%)
1	1/26/2018	A	*Cellana exarata*	1.20	15.43 ± 0.08
	GnRH Variant			Spawning Response (+/-)	
				Egg	Sperm
	Pheromone		Dilution (mg/L)		
		Control (FSW)	-	-	-
		sGnRHa	0.05	-	+
		sGnRHa	0.25	-	+
		sGnRHa	0.50	-	-
		olGnRH	0.05	+	-
		olGnRH	0.25	-	+
		olGnRH	0.50	-	+
	Injection		Concentration (priming, resolving - ng g^{-1} BW)		
		Control (PBS)	-	+	+
		sGnRHa	250, 500	+	-
		olGnRH	250, 500	+	+

As mentioned before, Hua and Ako (2013) found s-GnRH to function as a potential reproductive hormone for *C. sandwicensis*. In this study, the GnRH III analogue was found to stimulate gonad development and possibly induce spawning, perhaps indicating cross-reactivity with the native receptor or presence of a cognate receptor found in fish. However, this GnRH III analogue's alleged function is not necessarily representative of a putative GnRH V peptide predicted for limpets. Additionally, this teleost form lacks the addition of two aa in position 2 and 3 – believed to be a requirement for GnRH-like binding with cognate GnRH receptors (Sun *et al.* 2012). Furthermore, our phylogenetic analysis of GnRH primary structures – the only well-conserved regions of the metazoan prohormones – indicate that GnRH V are most similar to GnRH II. This is due to a homologous Gly-Trp motif that is hypothesized to be an ancient feature within the GnRH family (Tsai & Zhang, 2008). GnRH II is considered 'ancient' compared to those

functioning directly in reproduction (GnRH I/GnRH III) because it has been characterized from the midbrain – an ancient brain region – associated with basic sensory and motor function. Although lost or silenced in some mammals, this form is well-conserved in most chordates and may function as a neurotransmitter/neuromodulator. Given this relationship, GnRH V forms are likely to assume a similar functional role to chordate GnRH II – control of behavior.

Thus, it was no surprise that GnRH III and GnRH V forms appeared to have no effect on acute spawning in *C. sandwicensis* (see bioassay results in Table 5.3.). While considering our current findings are slightly contradictory to previous research, we argue that previous authors' conclusions were drawn prematurely.

Table 5.3. Two side-by-side bioassays investigating the effect of GnRH type III (sGnRH) and GnRH type V (olGnRH) on spawn induction of yellowfoot limpets (*Cellana sandwicensis)*. Wild, ripe animals ($N_{Exp.2}$=60, $N_{Exp.3}$=56) were collected from a rocky intertidal shoreline and spawned as individuals under stagnant conditions. Sex ratio (female to male) and gonadosomatic index (mean ± SD%) were determined prior to the experiment. All treatments were replicated by individual limpets for Exp. 2 (n=6) and Exp. 3 (n=8), respectively.

Experiment	**Date**	**Location**	**Species**	**Sex Ratio (f/m)**	**GSI (%)**
2	12/11/2018	B	*Cellana sandwicensis*	0.50	23.68 ± 8.35
	GnRH Variant			**Spawning Response (+/-)**	
				Egg	**Sperm**
	Pheromone		**Dilution (mg/L)**		
		Control (FSW)	-	-	+
		sGnRHa	0.05	-	+
		sGnRHa	0.25	-	-
		sGnRHa	1.25	+	-
		olGnRH	0.05	-	-
		olGnRH	0.25	-	-
		olGnRH	1.25	-	-
	Injection		**Concentration (priming, resolving - ng g^{-1} BW)**		
		Control (PBS)	-	-	+
		sGnRHa	250, 500	-	+
		olGnRH	250, 500	-	-
Experiment	**Date**	**Location**	**Species**	**Sex Ratio (f/m)**	**GSI (%)**
3	1/18/2019	B	*Cellana sandwicensis*	0.75	32.89 ± 5.63
	GnRH Variant			**Spawning Response (+/-)**	
				Egg	**Sperm**
	Pheromone		**Dilution (mg/L)**		
		Control (FSW)	-	-	-
		olGnRH	0.05	-	-
		olGnRH	0.25	-	-
		olGnRH	1.25	+	+
	Injection		**Concentration (priming, resolving - ng g^{-1} BW)**		
		Control (PBS)	-	-	-
		sGnRHa	250, 500	+	-
		olGnRH	250, 500	-	-

Our study, simply stated, provides additional contextual evidence that GnRH-like peptides do not stimulate acute spawning in gastropod molluscs. With that said, aside from spawn-induction, our findings do not negate GnRH III functioning as a peptide hormone to stimulate

gametogenesis (Hua & Ako 2013) or rule out behavioral roles (i.e. pre-spawn behaviors, feeding, etc) for putative limpet GnRH V.

In a one-of-a-kind study, GnRH peptides administered as pheromones induced acute-spawning dose-dependently in chitons (*Mopalia spp*.) (Gorbman *et al.* 2003). This spawn-induction method was never examined further nor applied in other invertebrate models, and authors at the time suggested for more research to 'confirm or deny' their unique findings. In a proximal gastropod cousin, *Aplysia californica*, GnRH-like receptors are highly expressed in osphradium – a peripheral chemosensory organ found in many molluscs – inferring a possible modal function of its ligand as a neurotransmitter (as opposed to hormone). When reviewing Prosobranchian gastropod chemosensory (Kohn 1961), we considered external chemosensory organs (i.e. osphradium, cephalic tentacles, and mantle tentacles) to be a possible binding-site for GnRH applied as a pheromone for *Cellana*. Therefore, despite that GnRH, introduced as a pheromone, failed to induce acute spawning, we shouldn't discount GnRH as a neurotransmitter binding to peripheral receptors. Again, furthering a speculative discussion towards non-reproductive functions for GnRH in Prosobranch limpets.

In contrast, physical injections (irrespective of treatment) appear to stimulate spawning (another confounding factor) and increases mortality by acute injury/stress. This is consistent with a study on tropical abalone (*H. asinine*), where 20% of both control groups (saline injection) spawned (Nuurai *et al.* 2010). For Hawaiian limpets *Cellana*, various techniques to procure gametes (i.e. dissection, peroxide, hormone control, bubbling) have been implemented, resulting in <15% spawn success for any method (Corpuz *et al.* 1983; Mau *et al.* 2018). *Cellana exarata* has also been reported to spawn around the new moon (Corpuz *et al.* 1983) though our laboratory has induced spawning outside of this window, repeatedly. Collectively, this indicates that for

Cellana, like Mediterranean limpet (*Patella caerulea*), non-invasive approaches are most practical for spawning limpets in aquaculture (Ferranti *et al.* 2018). Certainly, consideration of reproductive timing, size of animals, and physical/chemical interactions will be crucial for future spawn-induction experiments.

5.5. Conclusion

This was the first attempt to examine any reproductive effects of putative limpet GnRH-like neuropeptide *in-vivo*. The current study provides new considerations and future prospects to examining the endocrine system of Prosobranch molluscs. Findings from current research will further dialogue on the structures and functions of GnRH V isoforms. Moving forward, we suggest that molecular techniques (i.e. -omics approaches, immunohistochemistry, molecular biology) be applied to characterize native GnRH-like neuropeptides in *Cellana* spp., which has both biological relevance and commercial implications.

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Chapter 6

Title: Near-daily reconstruction of tropical intertidal SST from limpet shells to infer their growth rates

Abstract

Measurements of life-history traits can reflect an organism's response to environmental factors. In wave-dominated rocky intertidal ecosystems, measurements of key grazing invertebrates are constrained by extreme conditions. Recent research demonstrates mollusc shells to be high-resolution oceanographic climate proxies for SST as well as archival records of growth; however, no prior molluscan climate proxy has been demonstrated for the tropical rocky intertidal environment – a zone influenced by warmer waters, mixed tides, trade-wind patterns, and wave-action. Here, we show the first near-daily, spatiotemporal climate proxy for SST in the tropical rocky intertidal environment by coupling secondary ion mass spectrometry analysis of oxygen isotopes with the sclerochronology of *Cellana sandwicensis*, an endemic Hawaiian intertidal limpet, that is a significant biocultural resource harvested for consumption. We also develop a method for reliable interpretation of seasonal growth patterns and longevity in limpets. This study provides a robust approach to explore tropical intertidal temperature climatology and molluscan life-history.

6.1. Introduction

Understanding the phenotypic plasticity of marine species to environmental change is crucial to understand the dynamics of coastal populations. Recent responses across multiple marine taxa to extreme sea surface temperature (SST) anomalies highlight the need for intertidal research focusing on thermal tolerance and habitat shifts (Helmuth *et al.* 2006; Sanford *et al.* 2019). For rocky intertidal ecology, however, monitoring climate responses *in-situ* presents significant challenges under adverse tide-, wave-, and wind-exposed conditions; and sub-annually resolved oceanographic climate proxies have only recently been identified for this semi-terrestrial environment (Fenger *et al.* 2007; Prendergast et al 2013; Prendergast & Schöne 2017; Hausmann et al 2019). Similar to other oceanographic climate proxies (i.e. coral skeletons, fish otoliths, foraminifera), mollusc shell is precipitated in isotopic equilibrium with seawater (Epstein et al. 1953). In these accretionary hard-tissues, oxygen isotope ratio ($\delta^{18}O$) measurements can be aligned with physiochemical features to infer seasonality with more negative values reflecting warmer climate and more positive values reflecting cooler climate (Fenger *et al.* 2007; Surge *et al.* 2001; Schöne *et al.* 2002; Prendergast & Schöne 2017).

To date, robust reconstruction of seasonal to millennial sea-surface temperature (SST) from mollusc shells are numerous in mid-to-high latitudes – where $\delta^{18}O$ is strongly correlated with seawater temperature (Schöne *et al.* 2002; Burchell *et al.* 2013; Wang *et al.* 2012). In contrast, SST proxies in low latitudes are limited to subtidal organisms (primarily corals), as hydrological processes (i.e. rainfall, estuarian mixing) influencing sea surface salinity (SSS) can confound drivers of $\delta^{18}O$ (Corrège 2006). Therefore, while corals are established long-term proxies of mean annual SST (Dunbar *et al.* 1994, Linsley *et al.* 2000; Urban *et al.* 2000; DeLong *et al.* 2012), a high-resolution proxy for tropical intertidal climate is missing entirely.

Mollusc limpet shells are an excellent candidate for tropical intertidal climate proxy records due to their archaeological preservation, wide distribution, and sequential growth (Twadle *et al.* 2016). With absolute temporal alignment of growth, ecologists can interpret species' responses to physiological (i.e., ontogeny, reproduction) and environmental (i.e., extreme climate, tide) factors (Wefer & Berger, 1991; Schöne 2008; Gutiérrez-Zugasti *et al.* 2017).

Within the Hawaiian Archipelago, endemic intertidal limpets (*Cellana spp*.) are a significant biocultural resource declining in abundance and experiencing contracting population distributions (Bird *et al.* 2007). Due to complex and extreme rocky intertidal conditions, research on growth patterns of *Cellana* is limited (Kay *et al.* 1982, 2006).

To reliably reconstruct Hawaiian limpet growth patterns, we applied measurements of oxygen isotopes from secondary ion mass spectrometry (SIMS) from shell line and increment features formed during growth cessation (with represented time periods in parentheses): major growth line (annual cycle), minor growth lines (lunar cycle), and minor growth increments (tidal cycle). For these culturally and commercially important molluscan shellfish, resolving growth patterns and longevity has critical implications for aquaculture, conservation, and fisheries. Here we reconstructed the life-history of the yellowfoot limpet *Cellana sandwicensis* from three shells, two modern and one historical, by 1) developing the first near-daily spatial scale tropical intertidal climate proxy using SIMS analysis, and 2) determining seasonal growth and longevity. This study provides a robust approach to explore tropical intertidal temperature climatology and molluscan life-history.

6.2. Methods

6.2.1. Ecology of Yellowfoot limpet

In the Tropical Pacific, sympatric limpets (*Cellana melanostoma*, *Cellana exarata*, *Cellana sandwicensis*, *Cellana talcosa*) inhabit the Hawaiian rocky intertidal ecosystem, where they graze on crustose coralline algae (CCA) and epibenthic microorganisms. Distribution ranges from the splash zone (upper-intertidal) to subtidal zone, and across the entire Hawaiian Archipelago (Bird *et al.* 2007). They are dispersed across the majority of seamounts, atolls, and islands, however, not all species are present in every rocky intertidal locality, which reflects species-specific micro-habitat preferences.

The reproduction cycles for each species appears to vary in time and space, and on-going long-term monitoring efforts are in progress to define this critical life-history trait. Previous studies on the yellowfoot limpet *C. sandwicensis*, reveal that reproduction is highly synchronized from December to March (Kay *et al.* 1982; Mau *et al.* 2018). Gametogenesis also occurs from June to August, however, the level synchronicity and intensity of this second spawn period are inconsistent.

These limpets are gonochoristic and considered to be sequential hermaphrodites (Mau et al 2017). The sex ratio is near 1:1(M:F) during spawning season, however, we have directly observed populations to maintain disproportionate sex ratios.

Development of this broad-cast spawning limpet been described from egg to post-larvae, where settlement occurs in less than 4 days post-fertilization (Mau *et al.* 2018). This short larval

duration ensures recruitment to the same localized intertidal environment, and reduces likelihood of hybridization between sympatric species with similar life-histories (Bird *et al.* 2007).

For wild limpets, growth rates shift through ontogeny – average monthly growth decreasing from 4-5 mm shell length (SL) as juveniles to 2-3 mm SL as adults (Kay *et al.* 1982). Limpets also exhibit seasonal growth patterns – influenced by temperature and feeding (Kay *et al.* 2006; Mau & Jha 2018). Currently, growth rates of large individuals (>50 mm SL) and species longevity are absent in the literature.

6.2.2. Regional Climate and Coastal Oceanography

Ka'alawai is located on the south-facing shoreline of Oahu Island, Hawai'i (21°15'20.7"N 157°47'30.8"W). This area, defined as a rocky intertidal zone, is primarily comprised of basalt outcrops, boulders and benches, and supports a diverse community of epibenthic flora and fauna. The area is relatively easy to access by foot, and has been continuously exposed to various anthropogenic factors, which includes development, urban run-off, and subsistence fishing.

The microclimate of the region is characterized by mild, wet winters (January to March) and dry, hot summers (July to September). The mean daily atmospheric temperature range and mean daily sea-surface temperature range are 18.44-31.38 °C and 22.67-30.18 °C, respectively. The annual precipitation is low relative to windward sides of the island, with maximum rainfall of 6.35 cm (data sources: US climate station USC00519397: Waikiki 717.2; PacIOOS Nearshore Sensor 04 (NS04): Waikiki Aquarium). Although freshwater input from precipitation along this coastline is considered to be marginal, the mixing of submarine groundwater discharge generates a unique geochemical profile for surface seawater at Ka'alawai. In particular, the mean surface salinity for

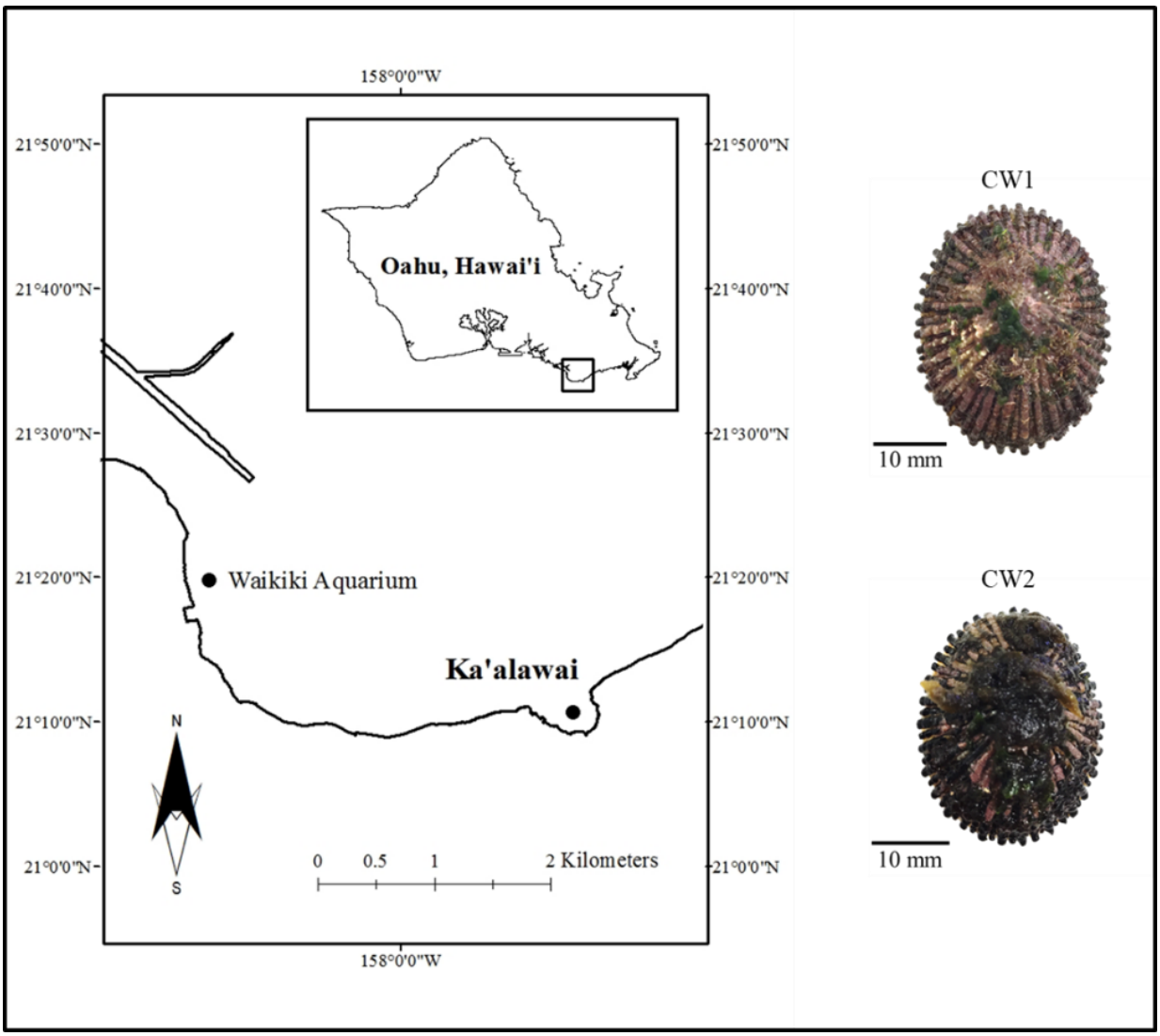
this study site has been reported to be 25.4 ‰, which reflects this highly localized land-sea interaction (Richardson et al. 2017).

The coastal oceanography of this region is predominantly influenced by wave, wind, and tidal forces. The south-shore region experiences a mixed tidal cycle – having both diurnal and semi-diurnal sinusoidal constituents per lunar day – with a tidal range of 58 cm and 91cm during neap tide and spring tide, respectively; The trade winds from north-easterly direction (between 22.5°-67.5°) account for ~63% of the year with mean annual intensity around 5 m/s (Garza et al 2012); and South swells with wave amplitudes of ~3 m are generated by storms in the Tasmanian Sea during Northern Hemisphere Summer (Snodgrass *et al.* 1966; Vitousek & Fletcher 2008).

6.2.3. Modern and Historical Specimens

On June 28th of 2018, live Yellowfoot limpet (*Cellana sandwicensis*) specimens CW1 and CW2 were collected from the rocky intertidal zone at Ka'alawai, Oahu, Hawai'i (Fig. 6.1.). The animals were immediately sacrificed/dissected using scalpel blade, and measured for shell dimensions using a caliper. Limpets were weighed to determine gonadosomatic index, and gonads were preserved for histological examination. Shells were rinsed in an ultrasonic bath and air-dried.

Figure 6.1. Hawaiian limpet specimens (*Cellana sandwicensis*) were collected along the rocky intertidal shoreline of Ka'alawai (Oahu, Hawai'i). Instrumental sea-surface temperatures were measured in-situ by PacIOOS Nearshore Sensor 04 (NS04) at the Waikiki Aquarium.



A historical specimen BPBM (identification number 250851-200492) was loaned from the Bernice Pauahi Bishop Museum Malacology Department Collection. This specimen's geographical and ecological origin is unknown, but was identified as *C. sandwicensis* by its characteristic shell morphology (Bird 2011). This specimen was selected for its large size to estimate life-expectancy of this limpet species, as well as to evaluate this method for paleoclimatology studies.

6.2.4. Characterization of Shell Microstructure

Shell microstructure was identified before isotopic analysis could be attempted. Each shell was cross-sectioned from anterior to posterior direction using a low speed saw (Isomet 1000, Buehler) equipped with a 0.5 mm diamond coated blade. Parallel cuts were made at the apex or maximal growth-axis to obtain two replicate 1.3 mm thick-sections per specimen. The first replicate thick-sections, prepared for micro-sampling, were further cut into <15 mm long pieces and mounted on a single glass round, and the second replicate thick-sections, prepared for sclerochronology, were mounted in its entirety on a large glass slide. Specimens were mounted in using quick-drying epoxy (EPO-TEK 301, Epoxy Technology Inc, Billerica, MA) set in a mold, grinded with F1000 grit SiC powder, and polished with 3 and 1µm Al_2O_3 powder on a lapping wheel. Polished sections on glass rounds were then sonicated, rinsed with methanol, and carbon coated to ~250Å (Cressington Carbon Coater 208carbon, Watford UK). Microstructures of unstained specimens were identified by Scanning Electron Microscopy (SEM; JEOL JSM-5900LV, USA) photomicrograph following MacClintock (1967).

Raman spectroscopy was used to characterize biogenic carbonate mineralogy by comparing shifts in relative peak position and intensity between calcite and aragonite polymorphs (Urmos *et al.* 1991) (Appendix B, Figure B.4.). A silicon wafer standard was used to determine spectral center when grating was 800 grooves/nm. Single spectrum analysis was performed in each microstructure layer using a green laser at 514 nm. A total of six (n=6) sampling sites were selected haphazardly for a given microstructure layer, which comprised of ten accumulations averaged across 10,000 seconds. The shell's exterior surface layer did not return clear spectral peaks, and thus has been excluded from our analysis.

6.2.5. Secondary Ion Mass Spectrometry Analysis

Hawaiian limpet *Cellana sandwicensis* shells CW1, CW2, and BPBM were analyzed for oxygen isotopes. Polished thick-sections were imaged by light and scanning electron microscopy (SEM) to guide sampling by ion microprobe. We sampled in the crossed-foliated, calcite layer (M+2) to avoid mixing calcite and aragonite layers, and sequential measurements were performed moving from the shell margin toward the apex along the growth axis.

To achieve sub-weekly resolution for an annual $\delta^{18}O$ cycle, modern specimens, CW1 and CW2, were analyzed using an average interval of 252 µm and 288 µm between samples, respectively. To achieve sub-annual resolution across multiple $\delta^{18}O$ cycles, the historical specimen, BPBM 250851-200492, was analyzed using an average interval of 554 µm. The total number of microprobe sample measurements was 55, 43, and 56 for CW1, CW2, and BPBM, respectively. A single, annual isotope cycle was analyzed for the modern specimens, and four annual isotope cycles were analyzed for the historical specimen.

The carbon-coated thick-sections were placed under vacuum conditions to prevent contamination prior to being measured by CAMECA-IMS-1280 ion microprobe (SIMS; W.M. Keck Research Laboratory, University of Hawai'i) for oxygen isotopes. The primary ion beam Cs+ was set at 2.5 nA for 120s presputtering. Ions were extracted at ~8 kV. Microprobe rastered across a 15 μm^2 area, which accounted for 1-3 lunar daily growth increments. Each measurement included 30 cycles with 10s integration period. The ^{16}O and ^{18}O were measured in multicollection mode using two Faraday cups with 10^{10} and 10^{11} Ω registers, respectively. The b-field was controlled by nuclear magnetic resonance. Mass resolving power was 1958 % min^{-1}, which allows detection of possible interference ions. To correct for instrumental isotope mass fractionation, University of Wisconson Calcite, UWC-3 ($\delta^{18}O$ = 12.49‰ Vienna Standard Mean Ocean Water – VSMOW), was measured consecutively before and after performing microprobe analyses for each specimen (n=12). The reproducibility measurements (2σ) of UWC-3 reference material ranged from 0.17 to 0.35‰, which reflected measurement precision and reproducibility of standard measurements for same-day measurements. Measurement errors are reported as 2σ, which reflects both precision (2 standard error) and reproducibility (2 standard deviation). Following the microprobe analyses, shell samples were images under SEM to expose sample scars for sclerochronology.

6.2.6. Predicted Shell $\delta^{18}O$

To examine if Hawaiian limpet shells are in isotopic equilibrium with their environment, we compared measured $\delta^{18}O_{calcite}$ to predicted $\delta^{18}O_{seawater}$ calculated from seawater-surface temperatures (SST). Sea-surface temperatures were obtained from in-situ PacIOOS Nearshore Sensor 04 (NS04): Waikiki Aquarium, Oahu, Hawai'i at 21.26587° N, -157.82275°W (Sea-Bird

Electronics model 37SMP, ±0.002 accuracy from -5 to 35 °C). Predicted values were calculated using the equilibrium fractionation equation for calcite and water described by Friedman and O'Neil (1977):

$$\textbf{Eq. 1}\ 1000\ln\alpha = 2.78 \times 10^6 / T^2 - 2.89$$

Where T is temperature in Kelvin and α is the fractionation between calcite and water from the equation:

$$\textbf{Eq. 2}\ \alpha = (1000 + \delta^{18}O_{calcite}\ (VSMOW) / (1000 + \delta^{18}O_{seawater}\ (VSMOW)$$

Conversion from VPDB to VSMOW scales were performed using the following equation (Coplen et al 1983):

$$\textbf{Eq. 3}\ \delta^{18}O_{calcite}\ (VSMOW) = (\delta^{18}O_{calcite}\ (VPDB) - 30.91) / 1.03091$$

The relationship between mean annual surface seawater $\delta^{18}O$ and mean annual surface salinity for the Tropical Pacific region (50-year data set) was used to calculate $\delta^{18}O_{seawater}$ (Legrande & Schmidt 2006):

$$\textbf{Eq. 4}\ \delta^{18}O_{seawater} = (0.201 \times Salinity) - 8.88$$

Mean surface seawater salinity was 25.4 ‰ for Ka'alawai (Richardson et al. 2017).

The temperature error for proxy measurements for CW1, CW2, and BPBM were ±1.54, ±1.53, and ±1.60 °C, respectively.

6.2.7. Temporal Alignment of Shell $\delta^{18}O$

Following SEM and SIMS, we removed the carbon coat with methanol and Kimwipe tissue. The same thick-sections analyzed by microprobe were stained following the previously mentioned procedure, and imaged by light microscopy. The SEM and light photomicrographs were over-layed using Photoshop to accurately expose the spatial-relationship between SIMS-analyzed points and growth lines/increments. For temporal alignment of isotope measurements, we used predicted $\delta^{18}O_{calcite}$ values that aligned with major lines as anchoring points. Calendar dates (based on a lunar year) were assigned for every isotope measurement between these anchors by counting micro lines (lunar daily growth). We applied previous research on growth and reproduction to resolve alignment discrepancies between predicted $\delta^{18}O_{calcite}$ and measured $\delta^{18}O_{calcite}$.

6.2.8. Climate Reconstruction of Historical Shell

The exact location from which the historical specimen BMBP was collected from is unknown. Climate was reconstructed from the shell isotope record – assuming that $\delta^{18}O_{calcite}$ is precipitated in equilibrium with $\delta^{18}O_{seawater}$. Sea surface temperature was calculated from sequentially sampled isotope measurements across ecologically relevant salinity values. Based on the profile with the most biologically relevant temperature thresholds (min and max), we predicted historical sea surface salinity (SSS).

6.2.9. Growth Measurements

The polished over-sized, thick-sections were stained with Mutvei's solution to expose major lines, micro lines, and micro increments by light microscopy (Schöne *et al.* 2005). Shell thick-sections were placed in a petri dish and submerged in Mutvei's solution for 45 minutes held constant at 37-40 °C with constant stirring. These stained thick-sections were imaged using Nikon Eclipse E600 Polarizing light microscope at 100x magnification for performing growth band measurements.

Daily growth was measured along two axes using the standard measuring tool in ImageJ. The first type of daily growth was measured between two micro increments along the growth axis. The second type of daily growth was measured along the horizontal axis (anterior to posterior orientation).

For the latter, we recorded x-coordinates for each point where a micro increment band intersected the M+3 layer, and subtracted x-coordinates of sequential points to calculate horizontal distance or growth. Back-calculated shell length measurements were used to model age-at-length data (see Appendix B).

We analyzed sub-monthly growth of modern specimens across an annual isotope cycle to understand temporal changes in growth. This period was selected for based on the shell length at which *C. sandwicensis* enters adulthood, which allows interpretation of adult growth (Kay *et al.* 1983).

6.2.10. Shell Growth Model

We used the back-calculated measurements of shell length-at-age as data inputs to estimate parameters of the von Bertalanffy growth function (VBGF), a standard method of describing growth in marine animals (Ricker 1975). The VBGF (Eq. 5) was fit to shell length (in mm) at age (in months) data for each shell individually (i.e., CW1, CW2, BH) and pooled samples using non-linear parameter estimation:

$$\textbf{Eq. 5} \quad L(t) = L_{\infty}\left(1 - e^{-K(t-t_0)}\right)$$

where $L(t)$ is length (mm) at age t (years), L_{∞} is the mean asymptotic length (mm), K is the growth coefficient, and t_0 is the theoretical age at length zero. Growth curves were fit by constraining L_0 to a common shell length of settlement in order to increase the accuracy of VBGF parameter estimates (Kritzer *et al.* 2001). The length at post-larval settlement (i.e., L_0) of *Cellana sandwicensis* was obtained from existing literature as 0.254 mm (Mau *et al.* 2018) and equated with t_0 = -0.09026. To determine if measurements from all shells could be pooled for a single growth model, we used likelihood ratio tests to test for pairwise differences in L_{∞} and K between shells by generating a χ^2 statistic for each set of comparisons (sensu Kimura 1980; Haddon 2011). Growth data for the pairwise comparisons were truncated to a shell length range of 0 – 45 mm that represented the range of data overlap for all three shells to minimize bias from the larger maximum size of shell BPBM (Kimura 1980). We used R v3.3.1 (R Core Team, Vienna, Austria), and Excel v2013 (Microsoft Corporation, Redmond, WA, USA) to perform the growth model statistical analyses.

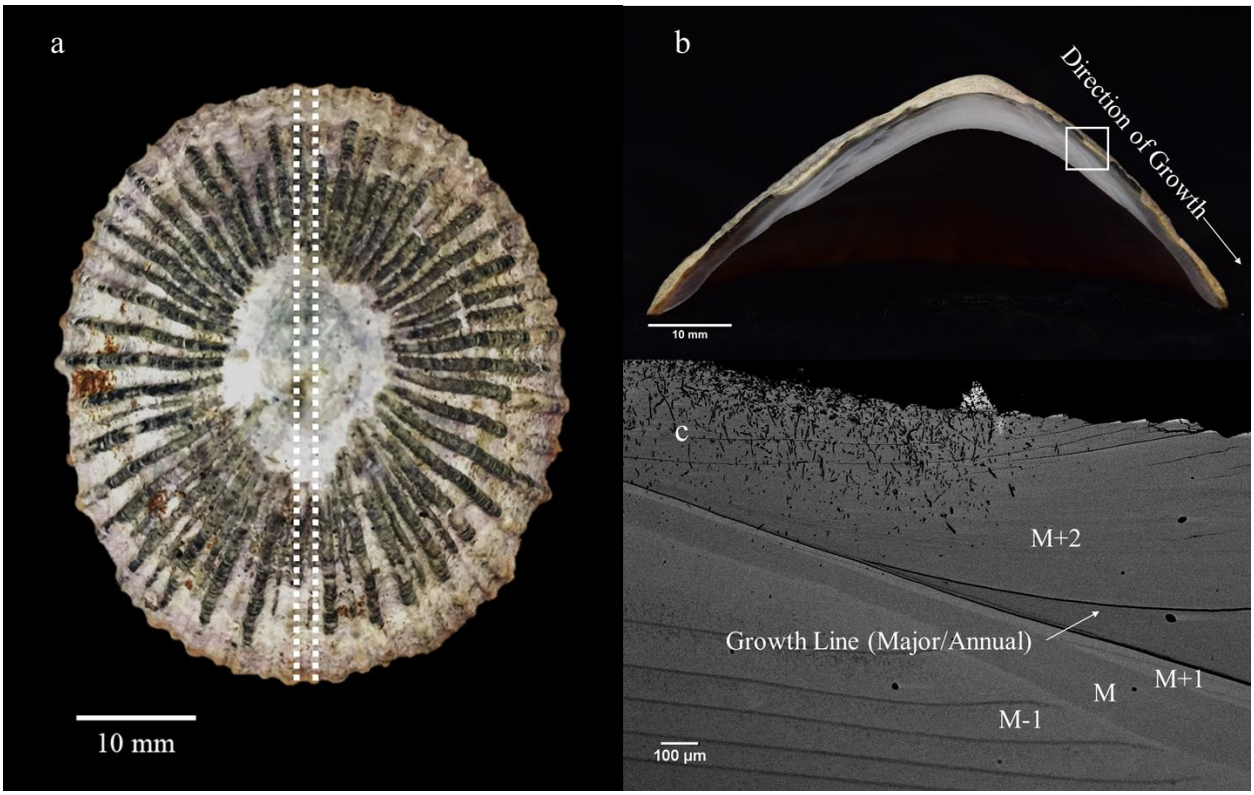
6.2.11. Statistical Analysis

Unless stated otherwise, all statistical analyses were accomplished in SAS (SAS v9.2, SAS Institute Inc., Cary, NC, USA). Pearson's correlation coefficients were computed using *Proc Corr* to determine linear relationships between $SST_{calculated}$ and $SST_{measured}$. We also used correlation analysis to describe monthly growth by changes in growth frequency and DG_{SL}, respectively. To analyze daily growth as a function of time, we performed univariate ANOVA with repeated measures using *Mixed Proc*. Pair-wise comparisons of unequal group sizes was performed using Tukeys-Kramer post-hoc analysis. Significant differences were determined using an alpha of 0.05 for all statistical procedures.

6.3. Results

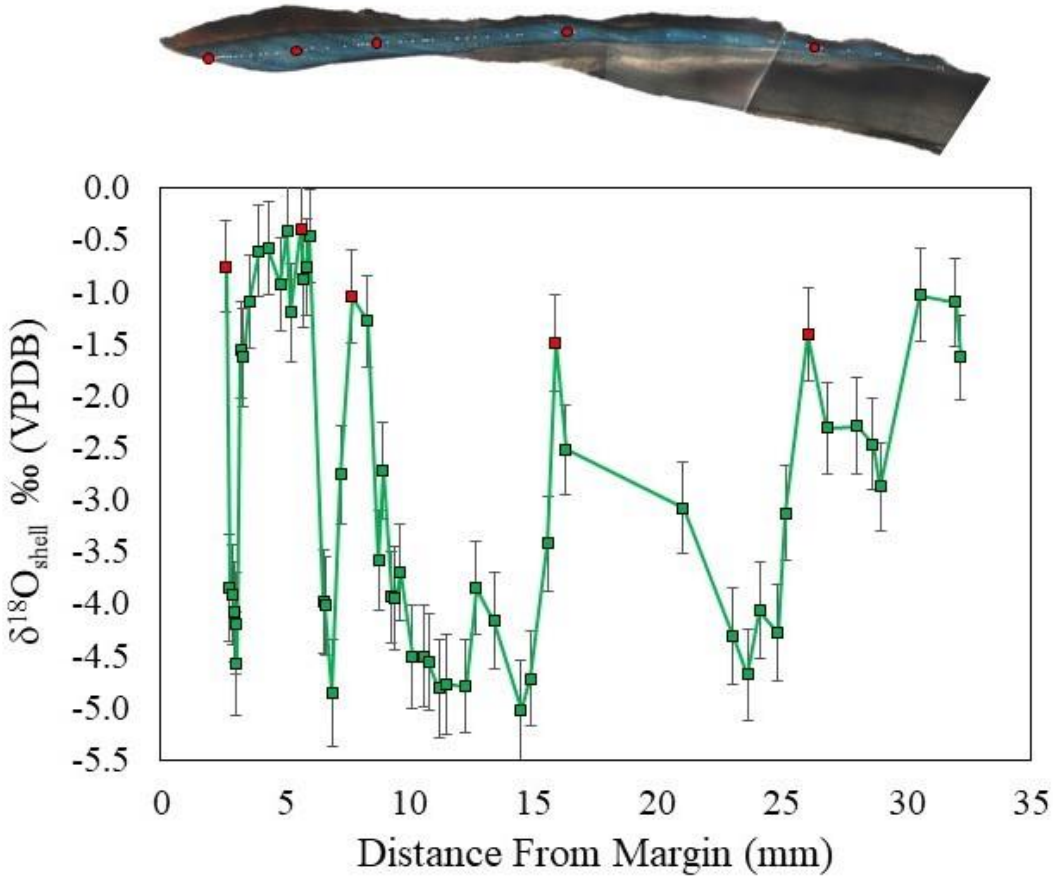
Within each of the three shells of *C. sandwicensis*, five carbonate mineralogical microstructure layers were revealed by SEM and Raman microscopy. With reference to the myostracum or muscle attachment layer (M), we observed one interior layer – aragonitic, radial crossed-lamella layer (M-1) – and two exterior layers – aragonitic, concentric crossed-lamellar layer (M+1) and calcite, concentric crossed-foliated layer (M+2), the latter being suitable for isotope measurements (Figure 6.2.). The carbonate polymorph was unidentifiable for the shell's outermost layer – a radial crossed-foliated layer (M+3).

Figure 6.2. Shells were sectioned from anterior to posterior end. A) White lines represent parallel cuts for thick-section preparation from historical specimen BPBM 250851-200492. B) Cross-sections show the true direction of growth for limpets. C) SEM exposed shell microstructures for area denoted in (B). Oxygen isotope measurements were performed along the direction of growth in the crossed-foliated, calcite layer M+2.



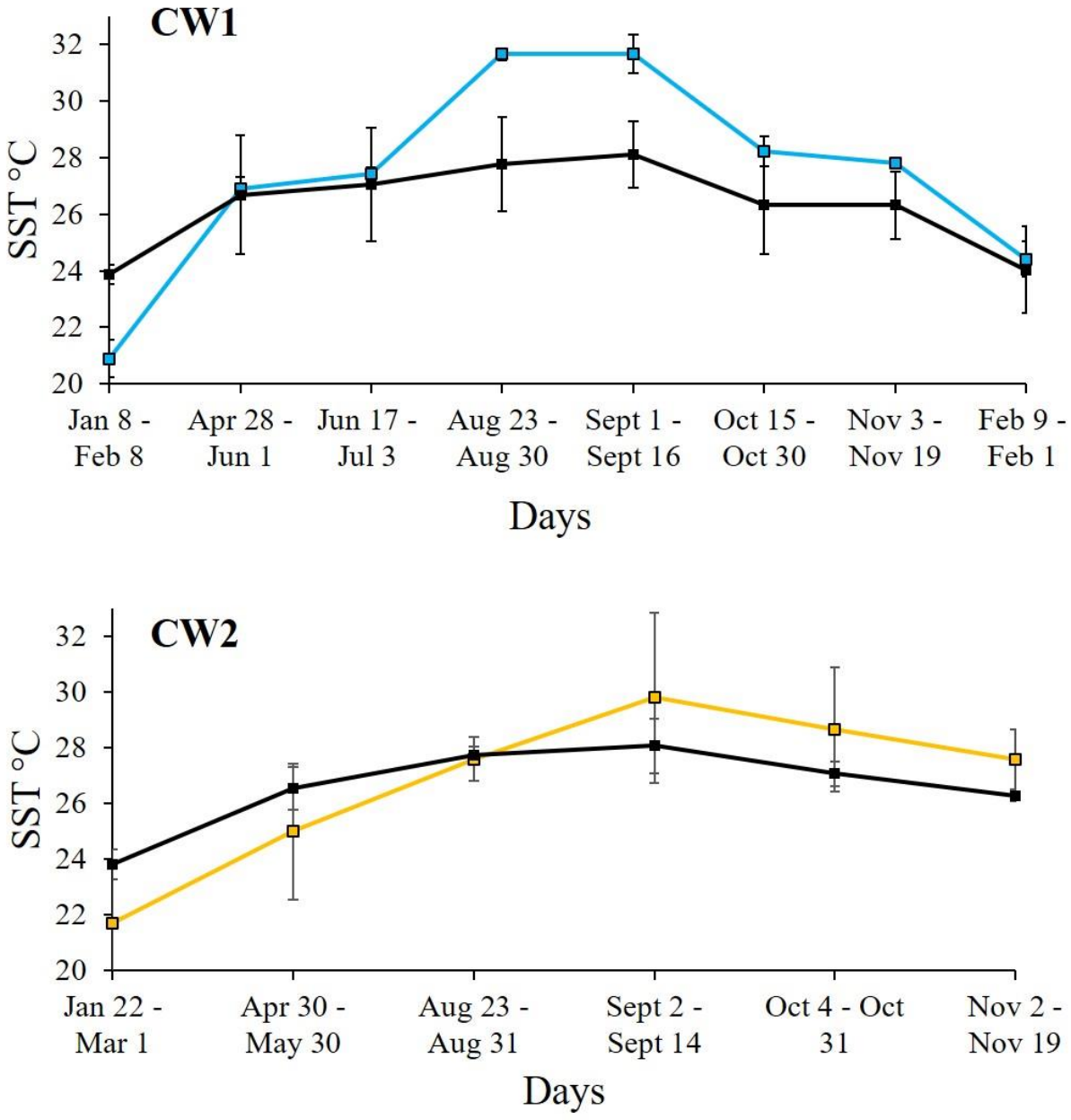
The $\delta^{18}O_{calcite}$ variation of three shells ranged from -5.04‰ to -7.74‰ (modern specimen CW1), -4.38‰ to -7.83‰ (modern specimen CW2), and -0.57‰ to -5.02‰ (historical specimen BPBM) (see Figure 6.3. and Figure B.4.). Across four annual isotope cycles, the BPBM oxygen isotope profile follows a sinusoidal pattern indicative of seasonal changes in the shell records of *C. sandwicensis*. Based on analytical precision (2 standard deviations) and measurement precision (2 standard errors), the maximum uncertainty for calculated SST from shell $\delta^{18}O_{calcite}$ was 0.51 ‰ or ±1.60 °C.

Figure 6.3. A shell cross-section and the associated oxygen isotope profile ($\delta^{18}O$) of a Hawaiian limpet (*C. sandwicensis*), reported in parts per mil (‰) relative to the international VPDB standard, were measured sequentially along the growth axis – starting at the shell margin. This pattern in the $\delta^{18}O$ profile of the historical shell (BPBM – green line) reflects the recorded seasonality in intertidal SST. The positive $\delta^{18}O$ measurements (red squares) were taken along and correspond with major bands (red circles) in the shell cross-section.



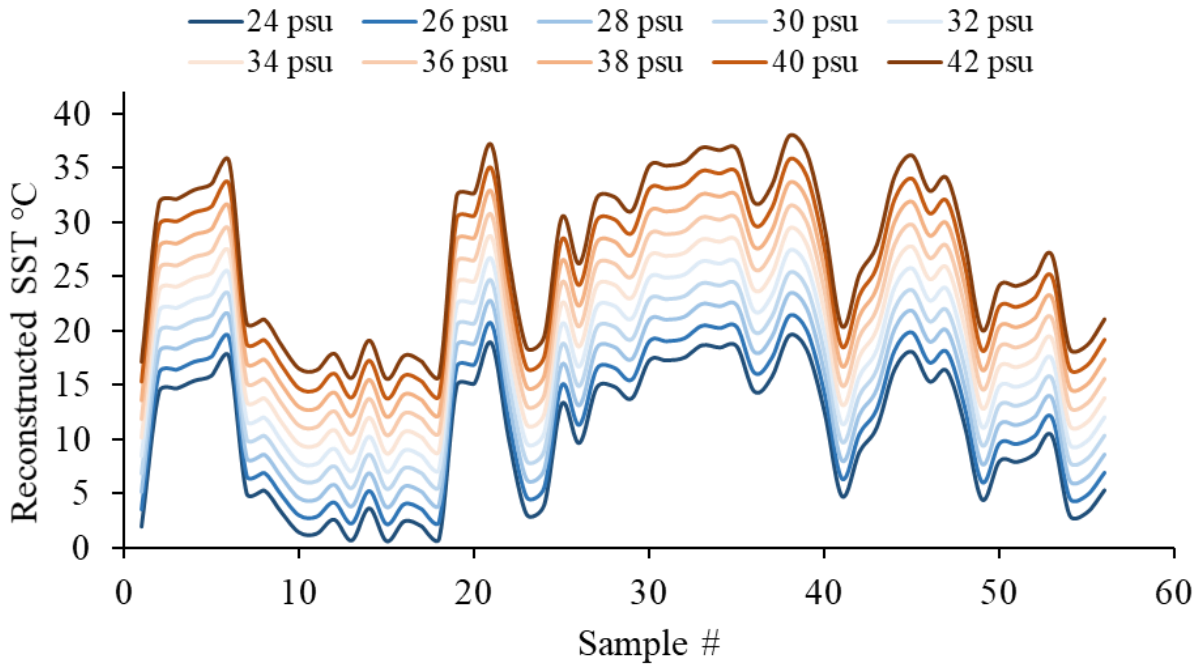
The correlation between proxy and instrumental SST measurements were strong and significant for both modern specimens (R^2=0.87; p=0.0007 and R^2=0.83; p=0.0113 for CW1 and CW2, respectively) (Figure 6.4.). The calibration of isotope data used in the paleo-temperature equation did not require off-set adjustments. For both shells, the median temperatures of proxy and instrumental data-sets were different by <1 °C. Proxy temperatures were overestimated (by up to 3.9 °C) and underestimated (by up to -3.0 °C) relative to SST, but were within the normal range for Ka'alawai's rocky intertidal substratum (see Supplementary Methods).

Figure 6.4. The comparison of proxy $SST_{calculated}$ (blue/yellow) and instrumental $SST_{measured}$ (black) showed a highly correlated and significant relationship for both modern specimens. Each data point represents the average SST for a given length of time. Error bars were calculated using the standard deviation of all reconstructed sample days in a period (n=3-12).



We modeled reconstructed SST profiles for the historical shell across a range of ecologically relevant salinity values and found an effect of evaporation on $\delta^{18}O_{calcite}$ (Figure 6.5). The reconstructed SST values changed by 0.84 ± 0.04 °C psu^{-1}. Temperature thresholds (T_{min} and T_{max}) were most biologically relevant at salinity of 42 psu. At 42 psu, SST ranged from 15.5 °C to 38.0 °C and averaged 27.1 ± 7.38 °C.

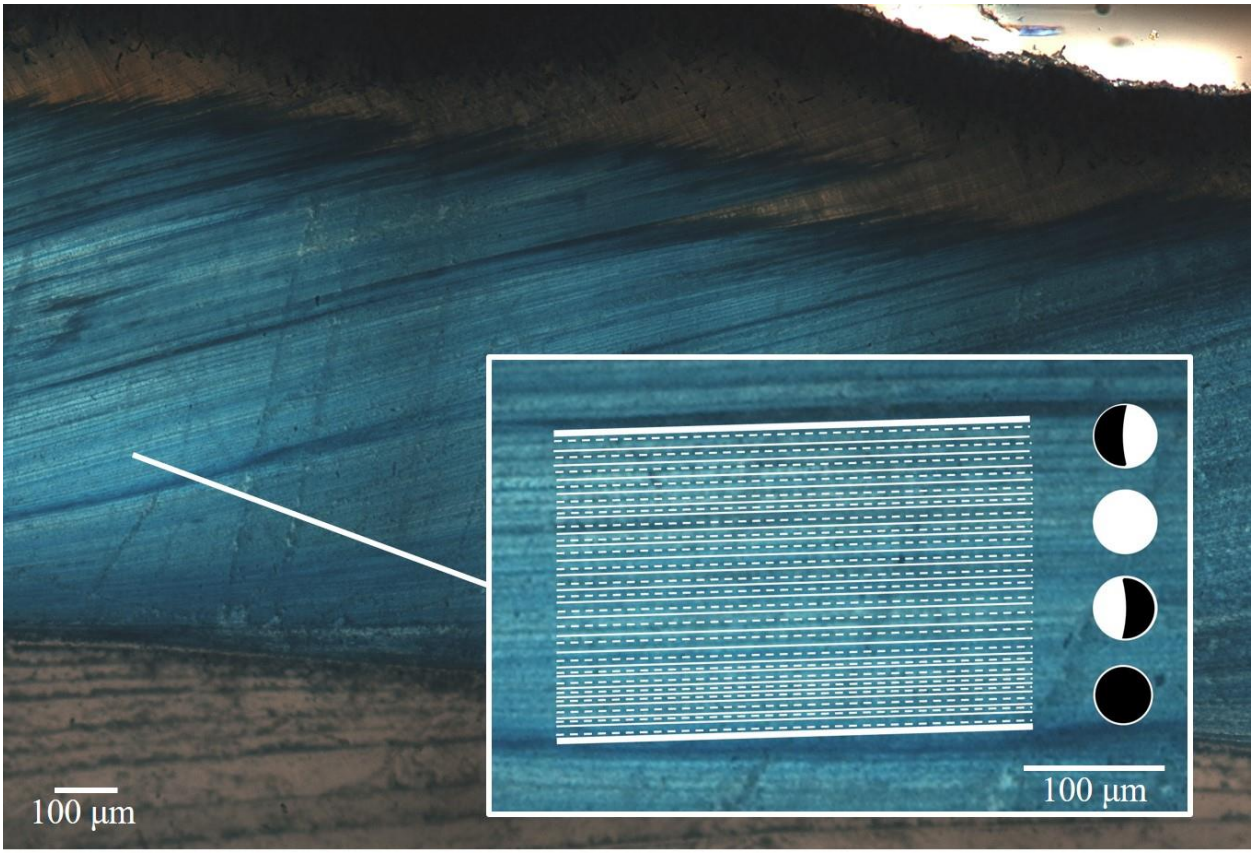
Figure 6.5. Reconstructed SST from historical specimen (BMBP) were modeled for ten different sea surface salinities assuming that $\delta^{18}O_{calcite}$ is precipitated in equilibrium with $\delta^{18}O_{seawater}$ for *Cellana sandwicensis*. Temperature thresholds were most biologically relevant at 42 psu. The sample number refers to sequentially and isotopically measured points in the direction of growth along the specimen.



The shell growth bands varied in physical appearance, which were correlated with environmental changes in the SST of the Hawaiian rocky intertidal (Figure 6.6.). The major-growth lines (annual) always corresponded to the most positive $\delta^{18}O$ measurements or lowest

temperature record (Figure 6.3). A major-growth line was also present in specimen BPBM – in alignment with the most negative $\delta^{18}O$ measurement. However, this major band was inconsistent through the multi-annual shell record and void in specimens CW1 and CW2. These major-growth lines were pronounced and spanned the width of M+2 layer, usually intersecting visible notches on the external surface of the shell. Minor-growth lines were observed in varying intervals of micro-growth increments (circalunidian); and micro-growth increments were subdivided by observed micro-growth lines (circatidal). The micro-growth increment widths ranged from 6.32 µm to 61.74 µm, but were typically less pronounced and wider for neap tides in comparison to spring tides, which were very pronounced and narrow. The average micro-growth increment widths for each specimen were 22.54 µm (CW1), 20.67 µm (CW2), and 13.69 µm (BPBM). The estimated age for modern and historical specimens was 2 years (CW1 and CW2) and 5 years (BPBM), respectively. These age estimates were based on the number of annual isotope cycles, and early ontogenetic growth recorded in the apex region (beyond the last isotope measurement).

Figure 6.6. Detailed growth features of *C. sandwicensis* shell that experience mixed tides in a wave-dominated rocky intertidal zone. Minor line appearance and periodicity was variable, and likely influenced by wave exposure (thick line). Micro lines were consistent and followed daily lunar cycles. We classified micro lines into two semi-lunidian increments (solid and dashed lines). During full and new moon phases, spring tides were recorded as prominent, narrow daily micro lines. During first and last quarter moon phases, neap tides were recorded as faint, wide microlines.



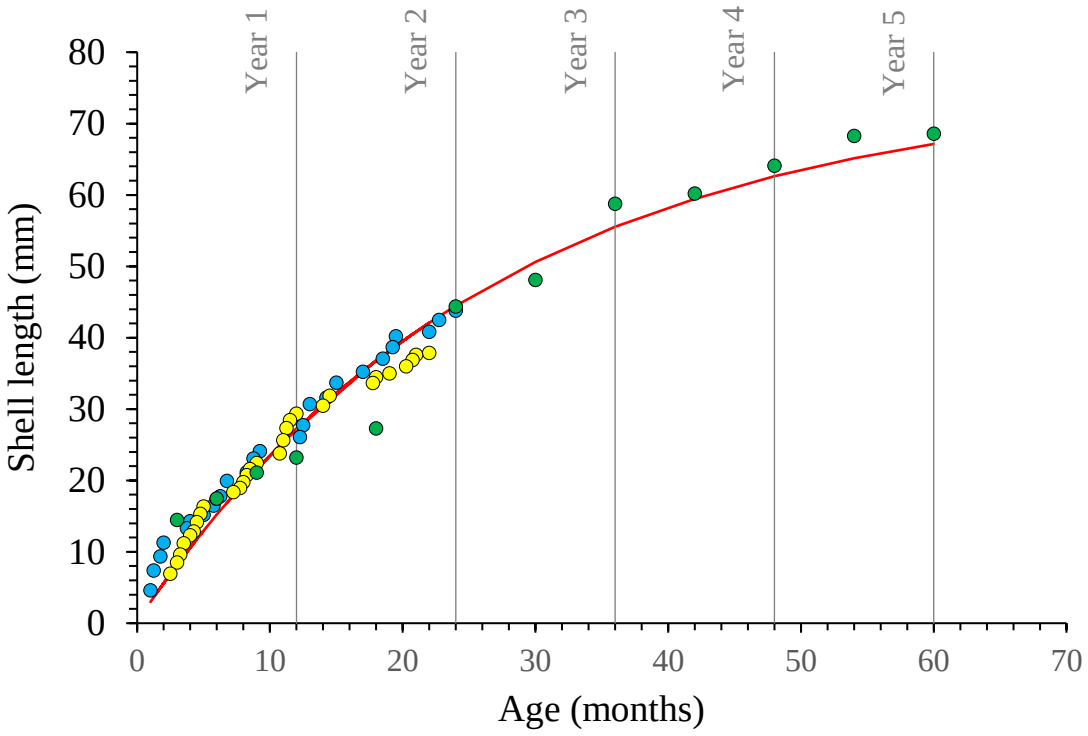
The back-calculated shell length measurements from calendar year 2017 were used to interpret sub-monthly growth rates of *C. sandwicensis* for the two modern specimens (see Appendix B, Table B.4.). Initial SL during this period were 20.75 mm (CW1) and 22.89 mm (CW2), respectively. Daily growth rates (DG_{SL}) ranged from -83 µm to 588 µm, and averaged 140 ± 93 µm (CW1) and 98 ± 95 µm (CW2), respectively. Across monthly observations, DG_{SL} was found to be highest in May for CW1 and January for CW2, respectively; and across seasons,

DG_{SL} was highest in Spring for CW1 and Winter for CW2, respectively (See Appendix B, Table B.1.). Total monthly growth ranged from 0 mm to 5.39 mm, and were significantly correlated with both growth frequency (R^2=0.81; p<0.0001; CW1 and R^2=0.67; p=0.0005; CW2) and DG_{SL} (R^2=0.61*; p*=0.0017; CW1 and R^2=0.89; p<0.0001; CW2), respectively. The total growth across calendar year 2017 for CW1 and CW2 was 19.96 mm and 15.01 mm, respectively. Modern limpets exhibited zero growth in both December and March, which lies in the primary spawning period. These limpets also exhibited zero growth during Summer (June-July for CW2 and August for CW1), which lies in the secondary spawning period. Furthermore, interrupted growth coincided with the most extreme temperatures calculated from oxygen isotopes.

For the historical specimen, the annual growth rate steadily decreased from 23.22 mm in the first year to 4.50 mm in the last year and averaged 13.83 ± 7.77 mm across a total of five isotope cycles. The average daily growth rate for BPBM was 81.78 ± 120.55 µm (see Appendix B, Table B.3.); and the maximum daily growth recorded was 605 µm. Monthly growth rates were not determined.

To compare shell growth rates, we modeled each of the three shells using a von-Bertalanffy growth function (VBGF). For the best fit VBGF model to pooled data from all three shells, parameter estimates were L_∞= 75.233 (±2.681 SE) and K = 0.0371 (±0.0021 SE) with t_0 constrained to represent an initial length of post-larval settlement of 0.254 mm observed for *C. sandwicensis* (Figure 6.7.; Mau *et al.* 2018). Modeled shell length at one year is 27.2 mm, two years is 44.5 mm, three years is 55.5 mm, four years is 62.6 mm, and five years is 67.1 mm. No differences in growth model parameter estimates between pairwise comparisons of individual shells were found for all χ^2 statistics (p > 0.05) from likelihood ratio tests.

Figure 6.7. Observed shell length-at-age measurements of *Cellana sandwicensis* (blue circles: modern CW1 shell; yellow circles: modern CW2 shell; green circles: historical BPBM shell) and the best fit von Bertalanffy growth model from pooled measurements of all three shells (red line).



6.4. Discussion

We used secondary ion mass spectrometry (SIMS) analysis to achieve sub-weekly to daily spatial scale resolution in the first tropical rocky intertidal climate proxy. Isotope measurements were extracted from an area of 15 μm^2 across the shell growth axis, which accounts for one to three growth days of any shell. To the best of our knowledge, this methodology represents the highest attainable spatio-temporal resolution for paleoclimatology.

The relationship between calculated and measured SST for modern shells validates *Cellana sandwicensis* to be a suitable climate proxy. Although the two shells showed some intraspecies isotopic variability, calculated SST fell within the expected range of temperature for the Hawaiian rocky intertidal shoreline.

There was a seasonal shift in the relationship between calculated and measured SST for modern specimens. We do not, however, totally discount that modifications in seawater chemistry of the extrapallial fluid are influencing calcification in *C. sandwicensis* (Langer *et al.* 2018); and vital effects (i.e. physiology, metabolism) are known to interfere with oxygen isotope fractionation that cause discrepancies in molluscan climate proxies (Schöne 2008). In future work, proxy calibrations to substratum temperatures will be useful to detect over- or under-estimation of climate.

To calculate oxygen isotope equilibrium fractionation (Eq. 1), we chose to apply an average salinity value for the study site; and because SSS and $\delta^{18}O_{seawater}$ are highly correlated, our resulting changes in $\delta^{18}O_{calcite}$ are driven by seasonal temperature fluctuations.

Given $\delta^{18}O_{seawater}$ was not measured directly, our maximum uncertainty of ±1.60 °C may be slightly underestimated as surface $\delta^{18}O_{seawater}$ is somewhat influenced by wet and dry seasons. We did not observe any major anomalies in the shell records. Rainfall events were previously thought to challenge interpretation of temperature derived from oxygen isotope profiles in tropical environments. We suggest that precipitation at both the modern shell (Ka'alawai) and the historical (unknown) sites occurs infrequently and the distance between isotope measurement locations were too far to detect these alterations in carbonate chemistry. Moreover, aside from rainfall and groundwater discharge, microhabitats within the same littoral zone vary tremendously with respect to evaporation, and are perhaps contributing to intraspecies variation

in carbonate chemistry. For instance, limpets inhabiting aerially exposed rock features may result in a wider $\delta^{18}O_{calcite}$ range than sub-surface limpets (Gutiérrez-Zugasti *et al.* 2017; Prendergast & Schöne 2017).

We reconstructed the past marine environment for an archival specimen (BMBP) by modeling proxy temperature regimes across a range of predicted salinities. We observe proxy temperature to approach a biologically relevant range at 42 psu. Given that our archival shell was recently collected (not part of a midden), the isotopic results suggest that both the $\delta^{18}O$ and SSS in the Hawaiian intertidal were possibly elevated by evaporation. At a salinity of 42 psu, we interpret this historical specimen's environment to be more aerially exposed compared to that of modern specimens, where extreme upper- and lower-threshold temperatures are likely a result of solar irradiance and evaporative cooling, respectively.

To address the precision of our inferences, we must consider that variability in the $\delta^{18}O_{seawater}$–SSS relationship may introduce error up to 1.5 psu in the Tropical Pacific (Stott et al 2004). Fortunately, when considering this relationship in the recent geologic time scale, there has been only marginal fluctuations (Stott et al 2004). Henceforth, our current methodology should also support sound paleoecology research using tropical archaeological middens dating as far back as early Polynesian settlement (AD 800) in the Hawaiian Islands. We discuss in further detail the thermal thresholds to growth, patterns in shell growth, and longevity of *C. sandwicensis* interpreted from the study.

6.4.1. Thermal Threshold

The thermal minimum and maximums observed vary by shell record, and thermal thresholds appear to change across time and space. Historical specimen BPBM recorded the widest thermal range, and did not grow below 15.5 °C or above 38.0 °C. Based on Hawaiian rocky intertidal substratum temperatures (15 °C to 40 °C), we understand this to reflect true thermal range for yellowfoot limpet (Bird 2006). Comparatively, modern specimens from Ka'alawai recorded lower threshold values suggesting that populations of *C. sandwicensis* may acclimate to their geographic and microhabitat environments.

6.4.2. Growth Patterns

Tropical limpets are exposed to mixed, semi-diurnal tides, which plays a role in growth band formation. Tide patterns allowed us to temporally align growth features around anchors (thermal min/max) similar to previous studies on temperate limpet species (Fenger *et al.* 2007; Prendergast & Schöne 2017). These patterns, however, were often interrupted at random causing inconsistent daily band widths. We attribute these inconsistencies to be influenced by waves, which dominate Hawaiian rocky intertidal shorelines (Bird *et al.* 2013).

Besides tidally influenced growth patterns, modern specimens exhibited a decrease in daily growth rate prior to complete stoppage in somatic growth in primary and secondary spawn periods, respectively. Although associated with spawning activity, it also appears that seasonal changes in growth rate are attributed to their response to desiccative conditions and temperature extremes (Fenger *et al.* 2007).

In a controlled study by Mau and Jha (2018), feeding, growth performance and mortality of *C. sandwicensis* were negatively affected by mean air temperatures >28.5 °C. These captive limpets also exhibited similar seasonal trends observed in CW2 – growth rates were lowest in Fall and highest in Winter – and further indicates that growth follows changes in climate.

All limpets in the current study exhibited determinate growth (Figure 7), where predictable changes in daily growth rate occur during ontogenetic shift from juvenile to adulthood (See Appendix B, Tables B.1. & B.2.). This type of growth, exhibited by yellowfoot limpet, is common in Tropical Pacific gastropods (Vermeij *et al.* 1992).

Age-at-maturity is an important metric for understanding population dynamics and assessment of managed stocks. For our isotopically analyzed yellowfoot limpet, age-at-maturity was 8-9 months (~21 mm shell length) which differed from a prior report of 4-7 months (Kay *et al.* 1981). The discrepancy between current and previous reports most likely arose because the previous authors assumed limpets <5 mm to be 1 month in age.

There was intra-species seasonal variability in growth, where highest mean daily growth rates were observed in May (CW1) and January (CW2), respectively. Frequency of growth, however, was consistently highest in Fall, which precedes the primary spawning window. Based on timing of maximal somatic growth and GSI, we understand that *C. sandwicensis* is likely bulking for reproduction twice, annually (see Appendix B, Eq. B.1. and Figure B.1.).

Monthly growth rates changed with frequency of growth days, and consistent growth cessation periods aligned with previously described spawning periods in both Winter and Summer months (longest period of missing growth was ~3 months). The differences in seasonal

growth patterns between individuals from the same population likely result from genetic variability (circadian rhythm) and environment (food availability and micro-habitat).

6.4.3. Longevity

Our sclerochronology of temporally aligned shell records provides the first reliable age estimate of *C. sandwicenesis*. The historical specimen represents the maximal size (68.6 mm SL), and indicates this species to live up to 5 years. The longevity of *C. sandwicensis* is similar to that reported for related species: *C. tramoserica* (3 yrs), *C. radiata* (4 yrs), *C. eucosmia* (5 yrs), and *C. karachiensis* (6 yrs), which indicates that *Cellana* is a relatively short-lived clade (Underwood 1975; Balaparameswara 1976; Emam 1994; Saad A 1997).

In theory, the longevity of marine gastropods is selected by environmental and biological pressures to ensure reproductive success (Powell & Cummins 1985). For *C. sandwicensis*, longevity may be influenced by wave exposure, average limpet size, population density, substratum type, and human harvesting. Current, ongoing research is focusing on these variables to evaluate their impact to the Hawaiian limpet fishery, and to establish adaptive management strategies.

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Chapter 7

7.1. Findings

Limpet aquaculture is an emerging area of research and development around the world. According to the current literature, reviewed in Chapter 1, abalone – a related intertidal gastropod mollusc – is the most suitable model for current and prospect technologies applicable to ‘opihi aquaculture; and from this literature review, target research areas have been identified for this dissertation: aquaculture systems and animal husbandry, feed and nutrition, and controlled reproduction and hatchery technology.

For marine limpets, including *Cellana sandwicensis*, nutritional requirements are not well understood, however, this is a significant component to animal husbandry and to effectively be able to produce this shellfish in the future. In Chapter 2, we examined the effect of protein to energy ratio diet on growth performance. We did this by staggering energy levels from carbohydrate sources with fixed protein levels of ~40% dry matter basis across four experimental diets (Diet1, 87; Diet2; 95; Diet3 97; Diet4, 103 mg/kcal) that were offered to ‘opihi held in a semi-indoor state-of-the-art rearing system. This study showed significant differences in average daily feed intake (ADFI) in periods which coincided with changes in temperature – an inverse relationship between temperature and ADFI – where feeding behavior was seemingly influenced by environment. There was a significant linear effect ($p < 0.05$) of dietary treatment on specific growth rate (SGR) at the end of the trial (d151-180), as well as across the entire study. The SGR of Diet 1 (0.12%BW/d) was significantly higher than that of Diet 4 (0.01%BW/d), indicating that a low PE ratio diet supports better growth than a higher PE ratio diet. This means that an increase in dietary energy from non-protein sources may free protein for tissue growth and weight gain.

In the subsequent study, Chapters 3 and 4, research focused on reproduction of limpets in aquaculture. Wild-collected limpets were maintained in maturation tanks, and for the first time, successfully matured limpets in captivity. In this study, broodstock gonadosomatic index (GSI) significantly increased between October and February, which mirrors the primary natural spawning window.

During the maturation trial, we also observed and documented the first hermaphrodite specimen for *C. sandwicensis*, and propose these limpets to be sequential hermaphrodites – providing a new theory for their shifting sex-ratio phenomenon (see Chapter 3). This finding was not conclusive by any means, but it supports a more plastic population and enhances species survivorship in adverse conditions.

After maturing limpets, ripe animals were injected with s-GnRH to induce spawning. Fecundity was linearly correlated with total body weight (including shell) (regression equation: $F=3007.8BW–14919$; $R^2 = 0.7934$; $p < 0.05$). Survival to pre-settlement competency was 35.4%, and competent veliger were then settled in petri dishes coated with natural biofilm. From egg to metamorphosis, there were 16 major larval development stages observed across 5 days.

After establishing baseline methodology for propagating ‘opihi, it was important to evaluate the effect of the gonadotropin releasing hormone on reproduction in our limpet model, as we were uncertain as to the efficacy of our hormone-control methodology. In Chapter 5, as part of advancing techniques for controlled propagation, we synthesized the putative owl limpet GnRH-like analogue (ol-GNRH) and tested the spawn-induction effects administered both as hormone and pheromone in side-by-side bioassays. Interestingly enough, both s-GnRH and ol-GnRH were found to have no direct spawn-induction effects on *Cellana* ($p > 0.05$). These semi-contradictory results are, however, in agreement with the latest research describing these

molluscan GnRH V hormones to stimulate non-reproductive, behavioral response. From a functional perspective, these findings highlight the capacity to continually spawn ‘opihi in spawning season. These results also indicate that implementation of natural spawning techniques can be effective, but require a level of awareness of the species’ natural reproductive cycles described in Chapter 4.

Lastly, in Chapter 6, we coupled sclerochronology and oxygen isotope chemistry to explore key life-history traits of *C. sandwicensis*. Following stable isotope analysis of the historical specimen (BMBP), the plotted oxygen isotope profile was found to follow a sinusoidal pattern, which is evidence for seasonality in the shell records of this species. Furthermore, the correlation between shell proxy and instrumental SST measurements were strong and significant for both modern specimens (R^2=0.87; p=0.0007 and R^2=0.83; p = 0.0113 for CW1 and CW2, respectively). By establishing this relationship, precise calendar dates could be assigned to each isotope sample point along the growth axis – resolving growth line deposition on a near-daily temporal scale. Based on temporal alignment of minimum and maximum temperatures, age estimates for modern and historical specimens are 2 years (CW1 and CW2) and 5 years (BPBM), respectively – the latter specimen resulting in the first longevity estimate for this species. To compare shell growth rates, all three shells were modeled using a von-Bertalanffy growth function (VBGF). *Cellana sandwicensis* was found to exhibit determinate growth, where age-at-maturity is just 8-9 month, and further supports the decision to select this candidate aquaculture species.

7.2. Conclusions

The overarching goal of this dissertation was to develop pilot-scale aquaculture technology for ‘opihi ‘alinalina. In reference to the overarching hypothesis, which states that *Cellana sandwicensis* can be cultivated in the laboratory environment, the major findings of this dissertation show that cultivation is indeed possible, but that more work is required to close the life-cycle of the species.

Following the literature review in Chapter 1, tests were conducted to determine if ‘opihi could be cultured in captivity. Although Chapters 2, 3, and 4 did not expand on the aquaculture system in great detail, a major component contributing to the success of this nutrition study was the design, construction, and implementation of the state-of-the-art aquaculture system. These studies showed that growing ‘opihi for a 6-month period (or longer) is possible, and that these limpets can be sustained on formulated feeds with similar growth to animals in the wild (see Chapter 2). Moreover, in Chapter 4, this same formulated feed appears to support good egg quality and meet nutritional requirements for limpets undergoing gametogenesis in the laboratory.

In previous work on *Cellana sandwicensis*, researchers were unable keep animals alive for more than a few months, which weakened authors’ interpretations. In Chapter 2, we maintained relatively high survival throughout the study, and this can be attributed to the design of the aquaculture system – supplied with underwater current (wave/tide surge), overhead spray (spray zone), and area to inhabit above the water-level as they do in their natural environment. This system also allowed for proper use of spatulas to transfers animals between tanks and spawn vessels without injuring them.

As far as the formulated diet and feeding regime, we established unique methods for limpet aquaculture by applying soft feed to the tank-surfaces at night. These limpets are considered nocturnal, most likely an adaptation to warm temperatures and predation forces during the day, and feeding them when the lights are off works well. The overhead spray was reduced to avoid feed-loss, but maintained "on" to keep the walls moist during feeding. This feed formula can be offered and/or supplemented when natural feeds are difficult to sustain growth in high-density production facilities.

In Chapter 3, we discuss implications for *C. sandwicensis* being sequential hermaphrodite, common for marine molluscs. This species' reproductive plasticity is not only fundamental to survivorship as a broadcast-spawning group, but also gives the cultivar confidence that their broodstock (sourced from either wild or captive populations) will regulate and/or maintain an appropriate sex-ratio for reproduction between seasons, naturally. Given external sexing of these limpets is nearly impossible, this reproductive strategy is advantageous to ensure good egg to sperm ratio (and reduce or eliminate polyspermy) during controlled spawn events. The limitation in this finding is that we report a one-time occurrence of hermaphroditism, which may have been an anomaly.

In Chapter 4, preliminary methodology for controlled-spawning, larval rearing, and settlement were developed. Through this study, it appears that current seed propagation is limited to their natural spawning season, but that individuals can spawn multiple times through this season. This species is also highly fecund, and based on the significant linear relationship between fecundity and body weight, which implemented the use of larger animals >40mm SL for seed propagation. According to results from Chapters 4 and 5, natural spawning techniques were both simple and effective, as well as less stressful on the animal. These limpets could be

spawned by simply placing them in stagnant (or bubbling water) as long as they reached final maturation. Although these findings challenge the use of hormone control, the natural spawning technique is viable and can be recommended for controlled spawning of limpets.

There are potential drawbacks to this spawning method when compared to artificial insemination, chemical induction (i.e. peroxide), and hormone control. This process is highly dependent on the reproductive status (stage of gametogenesis) of the individuals of a population, as our attempts to spawn individuals prematurely and/or at the end of the spawning season has resulted in low spawn frequencies (< 10% of population). Additionally, by following natural reproductive cycles, seed propagation will occur just two times per year (winter is the primary season and summer is the secondary season). This poses a potential bottleneck for aquaculture production.

Following these findings, we also described a shift in paradigm surrounding hormone-control spawning techniques previously implemented in ‘opihi aquaculture. We observed spawning patterns that appeared more or less random, and based on the positive spawning responses in many of our control vessels, it is probable that these hormones function to stimulate non-reproductive pathways such as to induce behavioral responses. Currently, research on the GnRH peptide hormone superfamily and the endocrine system of mollusc invertebrates using -omics approaches is a hot topic area of research. By understanding the structural and functional modalities of these peptide hormones, we may be able to enhance reproductive output and artificially control reproduction for year-round aquaculture production.

Lastly, moving into the final study, Chapter 6 describes the life-history of *C. sandwicensis*. In this study, a novel methodology to reconstruct sea-surface temperature in the tropical rocky intertidal environment was developed. The first longevity estimates (based on the

number of recorded isotope cycles) and growth curve were reported for the species. This ground-breaking life-history information improves our understanding of their life-cycle, and provides methodology to evaluate ‘opihi growth in captivity and in the rocky intertidal environment. By season, growth patterns in Chapter 2 and Chapter 6 were very similar, which again, supports the overarching hypothesis. In Chapter 6, growth of wild ‘opihi varied dramatically across seasons, and according to environment; and our ability to reconstruct environmental climate reveals temperature and salinity ranges and thresholds to consider in aquaculture.

7.3. Future Direction of Research

Throughout this dissertation, there were both examples of success and failure to support the overarching research hypothesis. To start, we have established some of the necessary, core components – multiple viable aquaculture systems, grow-out feed, animal husbandry protocols, natural spawn and larval rearing methodology – that support the culture of *Cellana sandwicensis* in controlled environments. We’ve also produced information related to the biology of the species that allows researchers to assess the candidacy of this species for aquaculture production. Metrics such as fecundity, age-at-maturity, growth curve, and longevity can guide expected production from the first ‘opihi farm. However, the focus of this dissertation was on pilot-scale studies to show that culture of limpets is truly possible. Through these studies, the culture of limpets in captivity has been shown to be possible, with identification of potential limitation and bottlenecks.

In the continuation of this work, research should focus on the settlement of ‘opihi ‘alinalina to close its life-cycle in aquaculture. The objectives in future work should be to

understand the mechanisms controlling settlement – the process of larval attachment to the benthos, and subsequent metamorphosis to juvenile stage – and to establish methodology for settling ‘opihi for junvile grow-out to market. In saying this, here are some of the preliminary findings regarding settlement of ‘opihi that were not mentioned in previous chapters.

Over the past three years of ‘opihi aquaculture research and development, multiple settlement trials have been conducted to examine various biological, chemical, and physical elements associated with settlement. For biological considerations, *settlement-competency* is defined as the ability for larvae to retract into their larval shell (closed operculum). Based on observations described in Chapter 4, the movement from water column to the benthos is “passive”, which means veliger larvae do not actively swim to the bottom, instead, their negative buoyancy, positive geotaxis, and/or negative phototaxis cause them to reach the benthos; for chemical considerations, various benthic-diatom-dominated biofilms were tested and appeared to influence metamorphosis after veliger larvae reach the benthos; and for physical considerations, substratum orientation (horizontal, slanted, and vertical plates) were tested, and horizontal plates were found to be most accessible for veliger larvae. Although these preliminary results were informative, they are, for the most part, inconclusive. The effects were too-often confounded with improper experimental designs and laboratory conditions (i.e. too much light intensity and light exposure, cold and fluctuating temperature, varying ages, qualities, and densities of biofilms, predatory zooplankton and ciliates, and human error). Moreover, all of these experiments resulted in population crashes by Day 14 post-fertilization.

In the most recent settlement trial, the goal was to surpass Day 14 post-fertilization, the point at which a major physiological phenomenon occurs (i.e. yolk-sac energy reserves become depleted). In this experiment, we examined the effect of the timing-of-exposure to substratum

signals on settlement by staggering the microalgae inoculation (*Navicula* sp.) in vessels as followed: 1 week pre-inoculated (control), Day 3-innoculated, and Day 7-innoculated. In the laboratory, environment was maintained at low light intensity <20 photons/m^2/sec with exposure of 5 hours per day, and stable room temperature of 23°C. Settlement vessels were filled with 1 µm-filtered, U.V. sterilized seawater with antibiotics prior to performing daily water changes.

In this experiment, ‘opihi were observed to metamorphose as early as Day 3 post-fertilization in both the control and Day 3-innoculated treatments. Interestingly enough, on day 3 of the experiment, the highest overall settlement success (up to 15% attachment and 11% metamorphosis) was observed in the treatment inoculated with microalgae that same day. This indicates that time-of-exposure to substratum cues may play a role in the settlement success. Surprisingly, survival of macroscopic juveniles reached 30 days post-fertilization (Figure 7.1.). It is suspected that biofilm quality and stability are critical to improving survival of post-larvae, and should be considered during future settlement experiments.

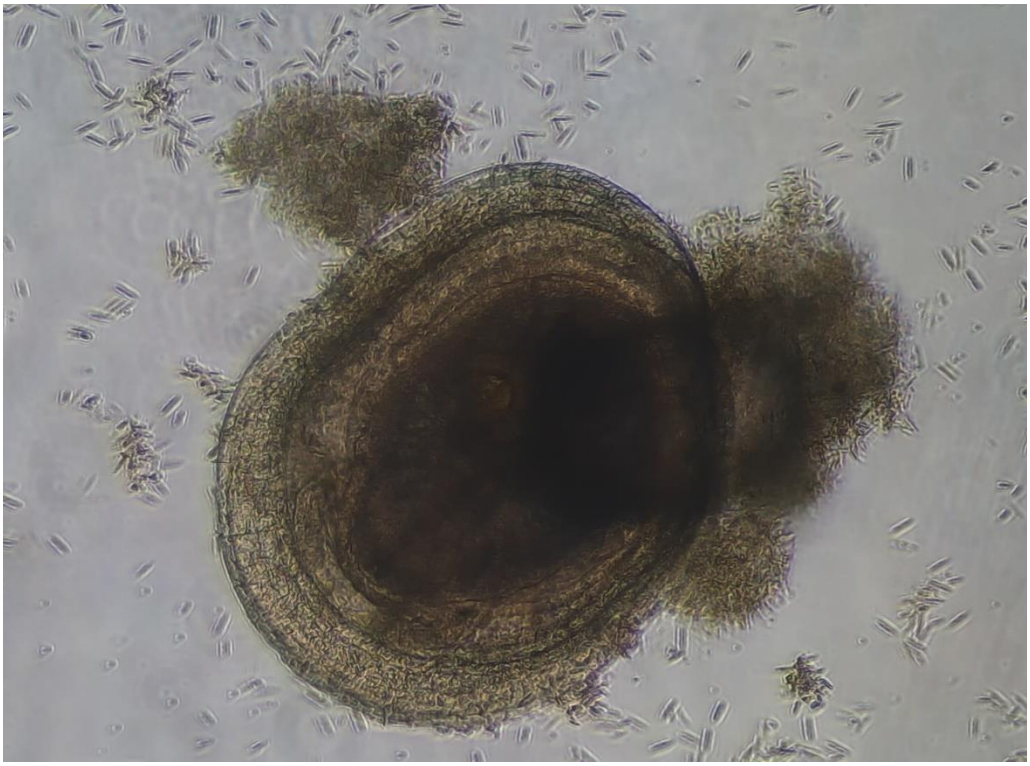


Figure 7.1. Post-settled ‘opihi ‘alinalina (*Cellana sandwicensis*) at 20 days post-fertilization was observed grazing *Navicula.*

Future research should focus on investigating chemical signaling that control early ontogenetic development, as the exposure to microalgae-dominated biofilm appeared to have an effect on settlement. This is not new, but for *Cellana sandwicensis*, there is no information pertaining to chemical induction of settlement. Some of the chemical inducers that should be examined for ‘opihi settlement are GABA, epinephrine, potassium chloride, serotonin, and acetal choline. These neurotransmitters have been found to modulate the pathway controlling larval invertebrate settlement in the marine environment, and understanding the biochemical pathway this fundamental process would improve the eventual navigation around bottle necks to production of limpets in aquaculture. The abalone industry, for instance, utilizes GABA to test

animals for competency before placing them in settlement tanks. For ‘opihi, a broad screening would suffice to direct additional studies on defining underlying mechanisms and pathways of these chemical inducers specific to *C. sandwicensis*.

If these bench-top experiments are successful, a scaled-up experiment would be required to increase juvenile production. We have recently designed and constructed a RAS settlement system with 9 raceways for these types of pilot-scale studies.

Lastly, established juveniles would need to be transferred to a nursery or grow-out system and weaned from biofilm to the formulated feed. At this stage of the research, growth rate and age-at-length data from Chapter 6 can be applied to determine stocking densities and monitor their growth. Restoration aquaculture and stock enhancement may be possible at this stage.

Overall, the future of ‘opihi aquaculture will depend on advancing the current hatchery technology. Progress must be made to control settlement and develop methodology for grow-out to market-size. From here, the final step would be economic analysis and business plan development for establishing the very first limpet farm: a significant milestone for the aquaculture industry.

Appendix A

Chapter 5 – Supplementary Section

Supplementary Methods

SPPS

Solid-phase-peptide-synthesis (SPPS) was performed manually using a 0.5 mM scale of amino acids on Rink amide resin (0.41 meq g^{-1}; Peptides International, USA) in a manual reaction vessel. The resin was swelled for 8-10 h in dimethylformamide (DMF; 25 mL; Fisher Scientific, USA). The Fmoc protecting group was removed via flow washing with DMF (1 min.), piperidine (1 min, x2, Alfa Aesar, Thermo Fisher Scientific, USA), and then washed with DMF (1 min.) prior to draining the manual reaction vessel. Each L-amino acid (Peptides International, USA) was activated by HBTU (4 mL; 0.5 M) and DIEA (347 uL, 2 mmol, Alfa Aesar, Thermo Fisher Scientific, USA) – employed as a proton scavenger. The activated amino acids were then added to the drained resin, and coupled for 10 min. A ninhydrin test was performed to ensure coupling yields reached 99.5%. In the case coupling yield was below 99.5%, additional milliequivalent amino acid was activated and recoupled. After passing with a satisfactory yield, the peptidyl-resin was subjected to repeated deprotection and amino acid activation-coupling cycles with adequate DMF flow washings. Side-chain protecting groups included: Ser(tBu), Lys(Boc), Trp(Boc), Gly, Asn(Trt), Phe, His(Trt), Tyr(tBu). After synthesis was completed, the peptidyl-resin was washed with DMF (5 mL) followed with Dichloromethane (DCM; 10 mL; Fischer Scientific) and dried under N_2 gas.

Cleavage

Cleavage was performed using modified Reagent K [TFA (82.5% v/v), phenol (5% v/v; Fisher Scientific, USA), water (5% v/v), thioanisole (5% v/v; Alfa Aesar, Thermo Fisher Scientific, USA), and triisopropylsilane (2.5% v/v TIPS; Alfa Aesar, Thermo Fisher Scientific, USA). The mixture was stirred for 2 h at 24 ℃, and filtered directly into liquid-nitrogen-chilled tert-butyl methyl ether (Fisher Scientific, USA). Peptide precipitate was pelleted by centrifugation (3000 g, 10 min.) and washed twice with chilled tert-butyl methyl ether. Resulting peptide pellet was suspended in 25% v/v acetic acid, freeze-dried, and stored at 4 ℃ until being chemically analyzed.

Supplementary Data

Figure A.1. Purified, synthetic olGnRH analyzed by RP-HPLC/UV at 214 nm. The resulting retention time was 30.7 minutes.

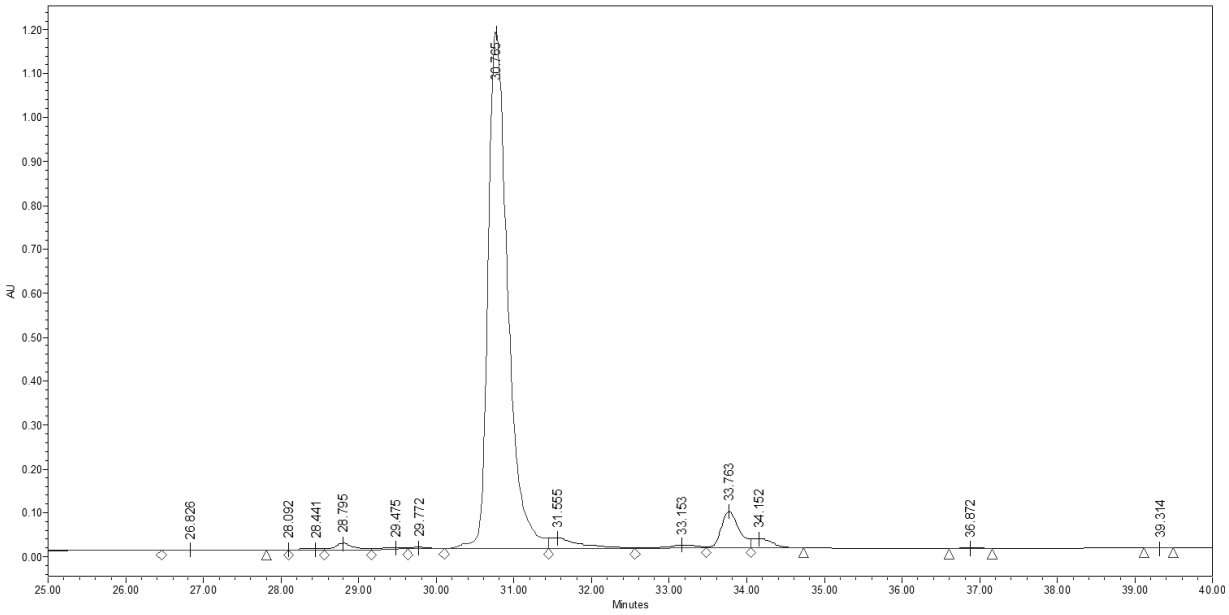
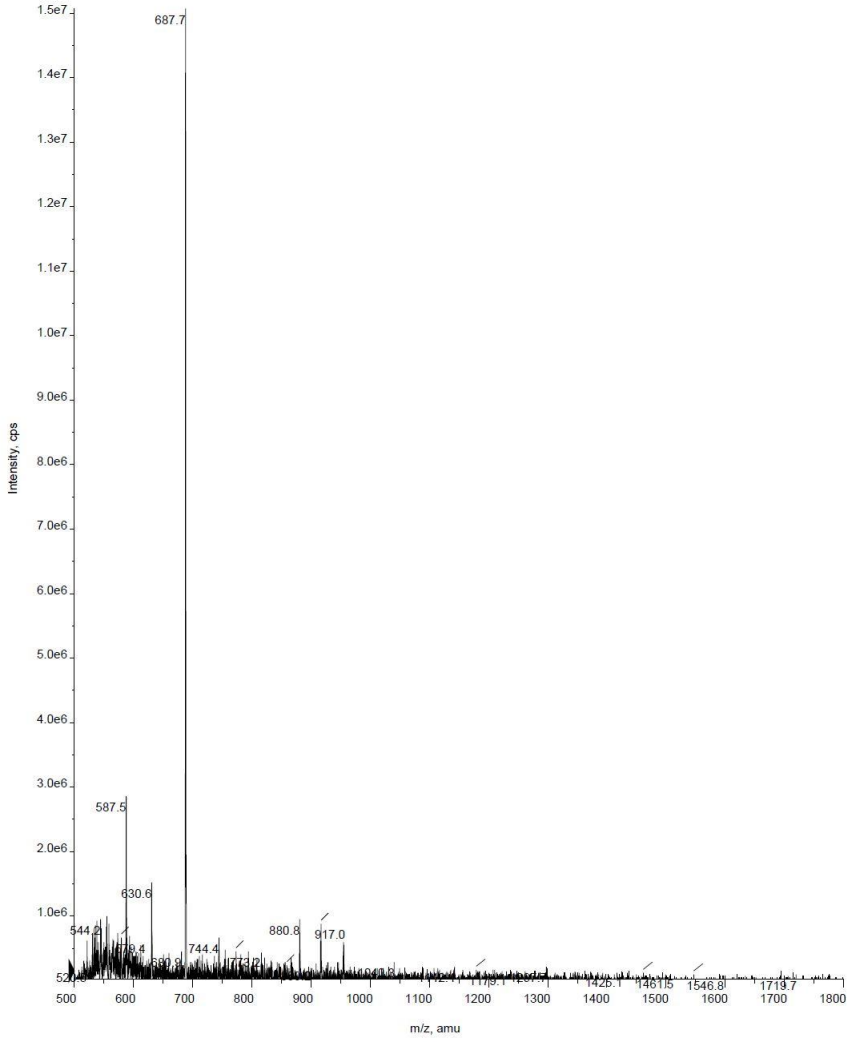


Figure A.2. Purified, synthetic olGnRH analyzed by ESI-MS. Mass spectrum of the chromatographic peak in Appendix Fig. 1 was used to confirm the molecular identity of synthetic olGnRH. The predicted molecular mass of oGnRH was 1372 Da. as a doubly charged molecular ion, and the synthetic peptide was observed at 687.7 Da.



Spawn induction experiments

Table A.1. Two additional pheromone bioassays investigating the effect of GnRH type V (olGnRH) on spawn induction of blackfoot limpets (*Cellana exarata*). Wild, ripe animals ($N_{Exp.4}$=40, $N_{Exp.5}$=60) were collected from a rocky intertidal shoreline, and spawned as individual limpets under stagnant conditions. All treatments were replicated by individual limpets for Exp. 4 (n=4) and Exp. 5 (n=6), respectively. We observed spawning in control groups for both experiments.

Experiment	**Date**	**Location**	**Species**	**Sex Ratio (f/m)**	**GSI (%)**
4	2/16/2018	A	*Cellana exarata*	-	-
	GnRH Variant			**Spawning Response (+/-)**	
				Egg	**Sperm**
	Pheromone		**Dilution (mg/L)**		
	Control (FSW)		-	-	+
	olGnRH		0.03	-	-
	olGnRH		0.15	+	-
	olGnRH		0.30	-	-
	olGnRH		0.65	+	+
Experiment	**Date**	**Location**	**Species**	**Sex Ratio (f/m)**	**GSI (%)**
5	3/2/2018	A	*Cellana exarata*	-	-
	GnRH Variant			**Spawning Response (+/-)**	
				Egg	**Sperm**
	Pheromone		**Dilution (mg/L)**		
	Control (FSW)		-	-	+
	olGnRH		0.15	-	-
	olGnRH		0.30	-	-
	olGnRH		0.60	+	+
	olGnRH		1.20	-	-

Appendix B

Chapter 6 – Supplementary Materials

Supplementary Methods

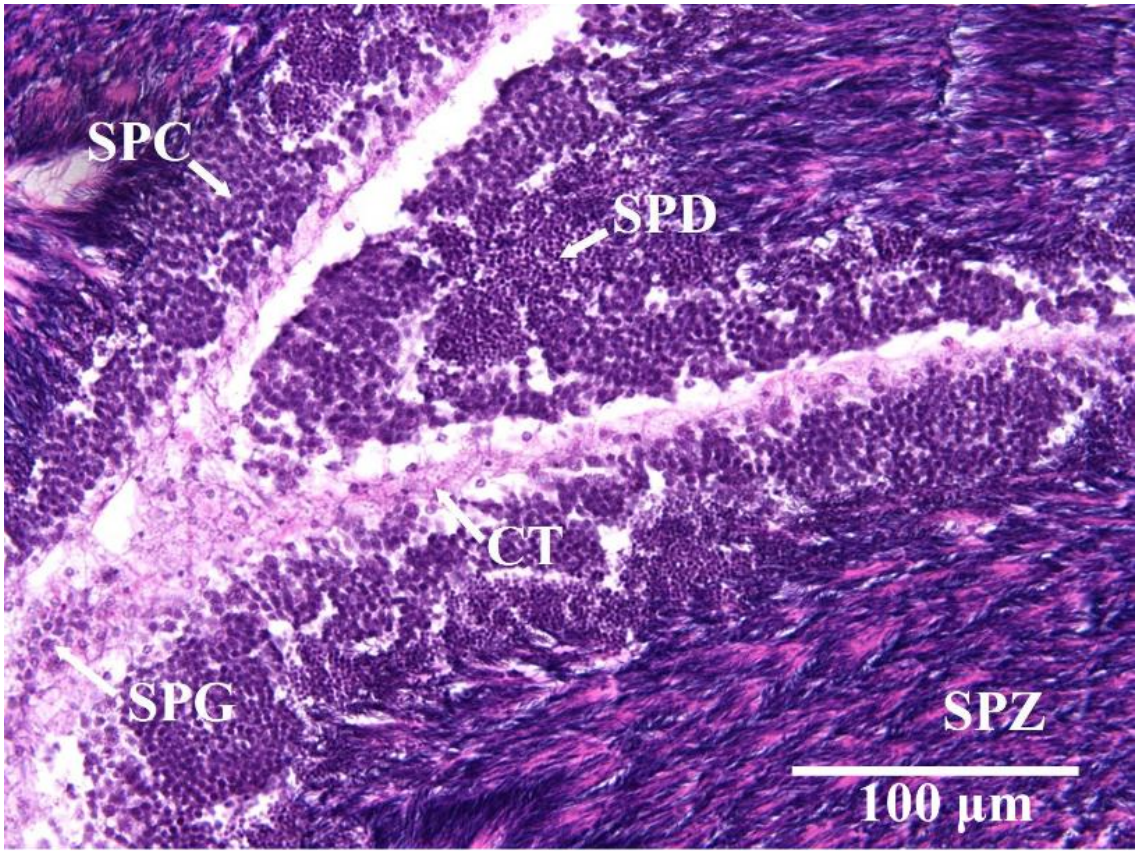
Characterization of Gonad

The gonadosomatic index (GSI), expressed as a percentage, was determined for freshly dissected specimens: CW1 and CW2:

Eq. B.1. Gonadosomatic Index (GSI)=gonad mass (g)/soft-body mass (g)*100

These gonads were then submerged in 10% v/v neutral buffered formalin for 24 hours at room temperature before transferring to 70% ethanol. The fixed gonads were wax embedded, and 6µm horizontal cross-sections were stained with haematoxylin and eosin at the Histology and Imaging Core Facility, John A. Burns School of Medicine, University of Hawai'i (supported by NIMHD, NIH grant # U54MD007601). Images were captured with Infinity Analyzed V 6.5.4 Lumenera Software and Infinity3S-1UR microscopy camera (Lumenera Corporation, Ottawa, ON), and Olympus BX43 (Olympus, Center Valley, PA) compound microscope. Gonad development was evaluated according to McCarthy et al. (2008).

Figure B.1. The histological examination from modern specimen (*Cellana sandwicensis*), CW1, collected on June 28th, 2018 at 40x magnification. The reproductive cells were observed to be present in multiple stages of maturation, indicating that the specimen was in late development – Stage 3 (McCarthy et al. 2008). Labels: spermatocytes, SPC; spermatogonia, SPG; connective tissue (trabecula), CT; spermatids, SPD; spermatozoa, SPZ.



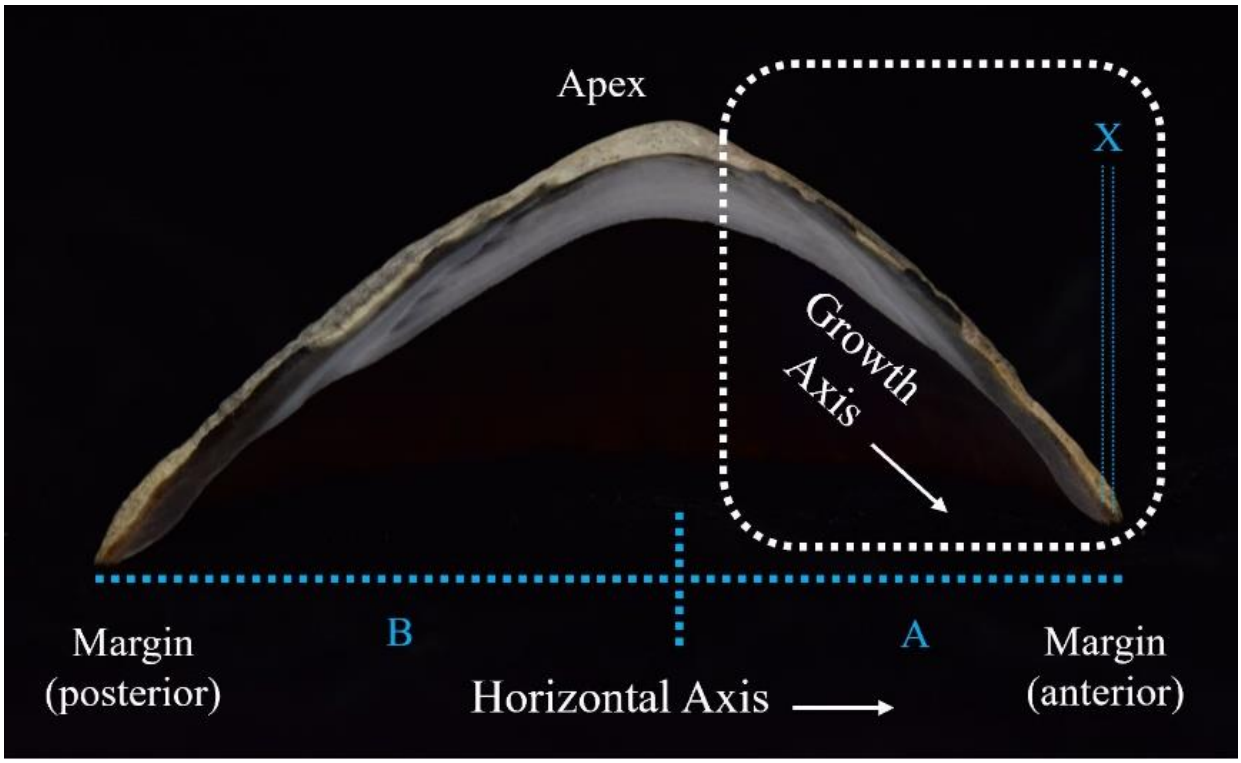
Growth Back-Calculations

Eq. B.2. Shell Length Increment ($SL_{increment}$)=X+X(A/B)

Where *x* is the difference in distance between adjacent micro-increment x-coordinates; *A* is distance along the x-axis from apex to anterior margin; and *B* is the distance along the x-axis from apex to posterior margin (Fig. 8).

We back-calculated the total shell length (SL_{total}) at respective growth increments starting from the margin and working towards the apex. We achieved this by subtracting the SL_{total}, which we measured via dial caliper, by $SL_{increment}$. This was repeated until all growth increments were given an associated shell length value.

Figure B.2. Diagram showing variables for Equation 4 used to back-calculate shell length measurements.



Intertidal Temperature

For proxy calibration, $SST_{calculated}$ was compared to SST measured via sub-surface logger fixed to the Waikiki Aquarium dock. This logger was chosen for calibration because of its relative

proximity to our study site and long-term deployment, however, the temperature range was narrow and does not reflect true substratum temperatures experienced by limpets.

To assess the accuracy of our proxy temperature records, our research team deployed a water-proof temperature logger (±0.2°C accuracy; HOBO MX2203, Onset Computers Corporation, MA, USA) at the mean low low-water (MLLW) height at Ka'alawai (where limpets were collected).

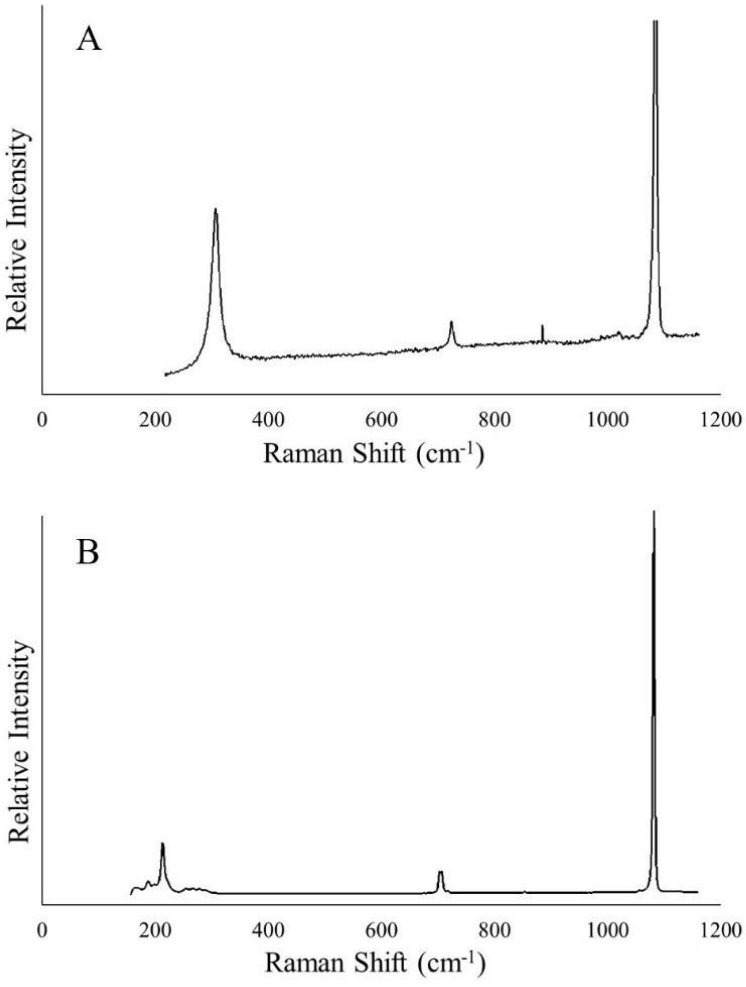
Ka'alawai's rocky intertidal substratum temperature ranged from 19.59 °C to 31.26 °C (between 03/30/18-03/30/19), and correlated strongly and significantly with the PacIOOS Nearshore Sensor 04 (NS04) Waikiki Aquarium logger (R^2=0.75; p<0.0001). The $SST_{calculated}$ values for modern specimens were within this substratum temperature range.

Figure B.3. The waterproof temperature logger was installed at the mean low lo-water (MLLW) of the low-intertidal zone at Ka'alawai.

Supplementary Data

Raman Spectrum for Biogenic Carbonate

Figure B.4. The Raman Spectrum of biogenic carbonate from Hawaiian limpet shell. Polymorphs Calcite (A) and Aragonite (B) were identified according to their relative Raman shifts for each microstructure layer



Proxy vs Measured SST

Table B.1. Summary statistics for $\delta^{18}O_{calcite}$ and sea-surface temperature in sequentially sampled specimen CW1.

Sample #	Date	$\delta^{18}O_{calcite}$ (‰ VPDB)	2σ	$SST_{calculated}$ (°C)	$SST_{measured}$ (°C)	Difference (°C)	2σ
1	3/1/2018	-5.77	0.42	24.23	25.10	-0.87	-1.26
2	2/28/2018	-5.04	0.43	20.83	23.20	-2.37	-1.31
3	2/27/2018	-5.86	0.43	24.63	23.80	0.83	-1.31
4	2/24/2018	-6.15	0.43	26.07	24.80	1.27	-1.31
5	2/21/2018	-5.97	0.38	25.16	24.30	0.86	-1.13
6	2/14/2018	-5.67	0.44	23.76	23.60	0.16	-1.35
7	2/12/2018	-6.07	0.50	25.66	24.00	1.66	-1.54
8	2/9/2018	-5.93	0.49	24.99	23.50	1.49	-1.53
9	11/19/2017	-6.03	0.48	25.46	26.03	-0.57	-1.44
10	11/16/2017	-6.52	0.47	27.86	26.35	1.51	-1.39
11	11/14/2017	-6.80	0.46	29.28	26.49	2.79	-1.48
12	11/9/2017	-6.55	0.42	28.00	26.28	1.72	-1.44
13	11/7/2017	-6.47	0.43	27.64	26.22	1.41	-1.40
14	11/3/2017	-6.67	0.42	28.59	26.50	2.09	-1.27
15	10/30/2017	-7.08	0.40	30.68	26.93	3.75	-1.31
16	10/26/2017	-6.87	0.41	29.58	26.14	3.44	-1.27
17	10/20/2017	-6.06	0.43	25.63	25.63	0.00	-1.20
18	10/18/2017	-6.43	0.40	27.43	26.06	1.38	-1.23
19	10/15/2017	-6.50	0.38	27.78	26.95	0.83	-1.31
20	9/16/2017	-7.13	0.42	30.91	27.99	2.92	-1.19
21	9/14/2017	-7.74	0.39	34.07	29.71	4.36	-1.11
22	9/12/2017	-7.10	0.38	30.79	27.35	3.44	-1.27
23	9/10/2017	-7.59	0.40	33.29	27.86	5.43	-1.14
24	9/8/2017	-7.17	0.40	31.11	27.66	3.45	-1.13
25	9/6/2017	-7.18	0.39	31.16	28.59	2.56	-1.18
26	9/4/2017	-7.12	0.40	30.87	28.02	2.85	-1.18
27	9/1/2017	-7.17	0.42	31.15	27.75	3.40	-1.16
28	8/30/2017	-7.43	0.43	32.48	27.82	4.66	-1.20
29	8/26/2017	-6.81	0.45	29.33	27.52	1.81	-1.26
30	8/23/2017	-7.57	0.41	33.16	28.00	5.16	-1.28
31	7/3/2017	-6.95	0.42	29.99	27.36	2.63	-1.36
32	7/1/2017	-6.45	0.42	27.50	26.71	0.79	-1.22
33	6/29/2017	-6.07	0.41	25.69	26.76	-1.07	-1.28
34	6/28/2017	-6.60	0.41	28.26	26.94	1.32	-1.28
35	6/26/2017	-7.33	0.42	31.95	27.24	4.71	-1.24

Sample #	Date	$\delta^{18}O_{calcite}$ (‰ VPDB)	2σ	$SST_{calculated}$ (°C)	$SST_{measured}$ (°C)	Difference (°C)	2σ
36	6/24/2017	-6.13	0.40	25.95	27.23	-1.27	-1.24
37	6/22/2017	-6.26	0.37	26.61	27.17	-0.56	-1.26
38	6/19/2017	-5.96	0.35	25.12	26.98	-1.86	-1.19
39	6/18/2017	-6.38	0.41	27.18	27.10	0.08	-1.09
40	6/17/2017	-6.18	0.39	26.20	27.10	-0.90	-1.00
41	6/1/2017	-6.78	0.36	29.14	27.10	2.04	-1.23
42	5/30/2017	-7.12	0.40	30.85	27.47	3.38	-1.15
43	5/29/2017	-6.75	0.39	28.99	27.06	1.93	-1.06
44	5/23/2017	-5.89	0.42	24.78	26.30	-1.52	-1.20
45	5/18/2017	-6.08	0.37	25.71	26.48	-0.77	-1.15
46	5/13/2017	-6.06	0.40	25.61	26.50	-0.89	-1.26
47	5/10/2017	-6.25	0.39	26.53	26.60	-0.07	-1.07
48	5/4/2017	-6.19	0.40	26.23	26.45	-0.21	-1.19
49	4/28/2017	-5.78	0.37	24.28	26.20	-1.92	-1.14
50	2/8/2017	-5.16	0.34	21.35	24.50	-3.15	-1.18
51	1/31/2017	-4.96	0.36	20.43	22.80	-2.37	-1.08
52	1/23/2017	-5.05	0.36	20.85	23.80	-2.95	-0.96
53	1/8/2017	-5.07	0.37	20.95	24.40	-3.45	-1.04
Mean		-6.41	0.41	27.39	26.35	1.04	-1.23
Max		-4.96	0.50	34.07	29.71	5.43	-0.96
Min		-7.74	0.34	20.43	22.80	-3.45	-1.54
Range		2.78	0.16	13.64	6.91	8.88	0.58

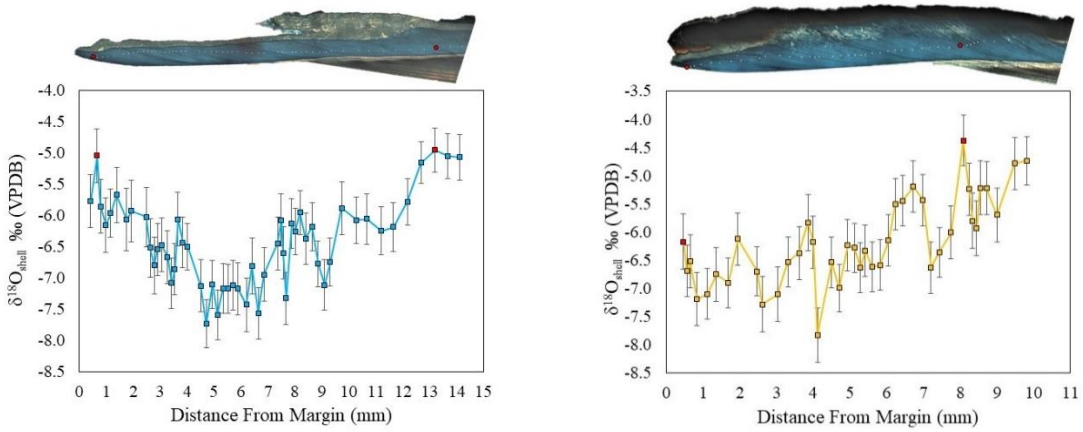
Table B.2. Summary statistics for $\delta^{18}O_{calcite}$ and sea-surface temperature in sequentially sampled specimen CW2.

Sample #	Date	$\delta^{18}O_{calcite}$ (‰ VPDB)	2σ	$SST_{calculated}$ (°C)	$SST_{measured}$ (°C)	SST Difference (°C)	2σ
1	11/19/2017	-6.17	0.49	26.17	26.03	0.14	-1.52
2	11/14/2017	-6.69	0.46	28.72	26.49	2.24	-1.41
3	11/11/2017	-6.52	0.47	27.88	26.37	1.50	-1.45
4	10/31/2017	-7.19	0.47	31.24	27.50	3.74	-1.45
5	10/30/2017	-7.10	0.45	30.77	26.90	3.87	-1.36
6	10/25/2017	-6.75	0.48	29.01	26.50	2.51	-1.48
7	10/22/2017	-6.90	0.44	29.76	26.90	2.86	-1.35
8	10/18/2017	-6.13	0.46	25.94	26.10	-0.16	-1.41
9	10/15/2017	-6.70	0.44	28.75	26.90	1.85	-1.34
10	10/13/2017	-7.29	0.49	31.72	27.60	4.12	-1.52
11	10/12/2017	-7.11	0.49	30.80	27.50	3.30	-1.51
12	10/8/2017	-6.53	0.44	27.93	27.40	0.53	-1.34
13	10/7/2017	-6.38	0.47	27.15	27.10	0.05	-1.45
14	10/6/2017	-5.83	0.49	24.53	27.00	-2.47	-1.53
15	10/4/2017	-6.18	0.46	26.22	27.60	-1.38	-1.40
16	9/14/2017	-7.83	0.49	34.55	29.70	4.85	-1.50
17	9/12/2017	-6.54	0.45	27.97	27.40	0.57	-1.38
18	9/7/2017	-6.99	0.42	30.24	28.00	2.24	-1.26
19	9/2/2017	-6.24	0.46	26.47	27.20	-0.73	-1.40
20	8/31/2017	-6.28	0.44	26.69	27.40	-0.71	-1.33
21	8/28/2017	-6.63	0.45	28.41	28.10	0.31	-1.35
22	8/25/2017	-6.33	0.46	26.95	27.40	-0.45	-1.42
23	8/23/2017	-6.62	0.44	28.36	28.00	0.36	-1.32
24	5/30/2017	-6.59	0.45	28.18	27.50	0.68	-1.37
25	5/28/2017	-6.15	0.46	26.05	26.60	-0.55	-1.39
26	5/26/2017	-5.51	0.45	22.99	26.40	-3.41	-1.38
27	5/23/2017	-5.44	0.45	22.69	26.30	-3.61	-1.37
28	5/16/2017	-5.20	0.46	21.55	26.10	-4.55	-1.39
29	5/10/2017	-5.43	0.46	22.63	26.60	-3.97	-1.41
30	5/5/2017	-6.63	0.46	28.41	27.80	0.61	-1.39
31	5/2/2017	-6.36	0.44	27.07	26.60	0.47	-1.33
32	4/30/2017	-6.01	0.48	25.36	25.00	0.36	-1.47
33	3/1/2017	-4.38	0.45	17.82	23.30	-5.48	-1.37
34	2/28/2017	-5.24	0.47	21.75	23.80	-2.05	-1.43
35	2/26/2017	-5.80	0.49	24.38	24.20	0.18	-1.52
36	2/20/2017	-5.94	0.49	25.02	24.40	0.62	-1.51
37	2/12/2017	-5.22	0.48	21.64	24.00	-2.36	-1.47
38	2/11/2017	-5.23	0.47	21.67	24.00	-2.33	-1.44

Sample #	Date	$\delta^{18}O_{calcite}$ (‰ VPDB)	2σ	$SST_{calculated}$ (°C)	$SST_{measured}$ (°C)	SST Difference (°C)	2σ
39	2/8/2017	-5.70	0.48	23.88	24.50	-0.62	-1.47
40	2/1/2017	-4.78	0.46	19.65	23.30	-3.65	-1.40
41	1/22/2017	-4.74	0.42	19.43	22.80	-3.37	-1.28
Mean		-6.18	0.46	26.25	26.35	-0.09	-1.41
Max		-4.38	0.49	34.55	29.70	4.85	-1.26
Min		-7.83	0.42	17.82	22.80	-5.48	-1.53
Range		3.45	0.07	16.73	6.90	10.33	0.27

Oxygen Isotope Profiles of Modern Specimens

Figure B.5. The oxygen isotope profiles ($\delta^{18}O$) of modern Hawaiian limpet specimens (CW1 – blue and CW2 – yellow), reported in parts per mil (‰) relative to international VPDB standard, were measured sequentially along the growth axis – starting at the shell margin. The positive $\delta^{18}O$ measurements (red squares) align with major bands (red circles) of each shell.



Growth Pattern

Table B.3. The growth patterns, based on lunar days, were determined for sequentially sampled shell specimens (n=3) according to major developmental stages (juvenile<21 mm SL; adult>21 mm), and the entire shell record (lifetime). Daily Growth = DG_{SL}, mean ± standard deviation.

Growth Period	**Life-time Growth (days)**		
	CW1	CW2	BPBM
Juvenile	157	154	55
Adult	192	162	788
Lifetime	349	316	843
	Time Growing (%)		
	CW1	CW2	BPBM
Juvenile	66.5	58.0	62.2
Adult	40.7	42.2	52.4
Lifetime	49.3	48.7	47.6
	DG_{SL} (µm day^{-1})		
	CW1	CW2	BPBM
Juvenile	150.70±126.67	103.65±67.64	167.43±154.82
Adult	134.63±93.91	98.94±93.57	71.07±111.00
Lifetime	140.30±93.06	98.13±90.29	81.78±120.55

Table B.4. Summarized sub-monthly growth analysis for adult *Cellana sandwicensis* modern specimens CW1 and CW2. The growth frequency, daily growth (DG_{SL}), and Total Growth are reported for Calendar Year 2017. DG_{SL}, mean ± standard deviation. Groups with the same letters are not significantly different (p>0.05).

	Growth Frequency (n)		**DG_{SL} (µm day^{-1})**		**Total Growth$_{SL}$ (mm)**	
	CW1	CW2	CW1	CW2	CW1	CW2
Month						
January	26	25	127.91±100.32^{abc}	149.09±102.94^{c}	2.05	2.68
February	11	31	107.90±59.65^{abc}	128.26±91.42^{bc}	0.86	3.08
March	-	-	-	-	-	-
April	7	11	167.44±111.57^{abc}	37.49±35.81^{abc}	1.17	0.26
May	31	18	207.41±120.44^{c}	131.68±101.29^{bc}	5.39	1.98
June	24	-	123.55±69.96^{a}	-	2.84	-
July	14	-	109.03±58.79^{a}	-	1.53	-
August	-	13	-	126.44±74.80^{bc}	0.00	1.52
September	12	16	155.08±71.73^{abc}	75.91±85.18^{abc}	1.71	1.29
October	14	31	107.74±61.96^{abc}	62.98±73.01^{ab}	0.75	1.45
November	25	17	116.76±66.72^{a}	1.14±34.53^{a}	2.92	0.01
December	-	-	-	-	-	-
Season						
Fall	51	64	125.09±69.63^{a}	56.11±77.15^{a}	5.38	2.75
Winter	37	56	121.24±89.36^{a}	137.19±97.07^{b}	2.91	5.76
Spring	38	29	198.93±119.73^{b}	101.71±96.58^{ab}	6.56	2.24
Summer	38	13	118.06±66.33^{a}	126.44±74.80^{ab}	4.37	1.52
Year						
2017	164	162	140.30±93.06	98.13±90.29	19.22	12.27